MECHANISMS INVOLVED IN EXOCRINE PANCREATIC SECRETION

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PROMOTOR:

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Mechanisms involved in exocrine pancreatic secretion

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Pa mi mayores nan
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CONTENTS

ADP	Adenosinediphosphate
AMP	Adenosinemonophosphate
ATP	Adenosinetriphosphate
ATPase	Adenosinetriphosphatase
cyclic AMP	Adenosine 3',5'-monophosphate
EDTA	Ethylenediaminetetraacetate
GTP	Guanosinetriphosphate
ITP	Inosinetriphosphate
P_i	Inorganic phosphate
Tris	Tris-(hydroxymethyl)-aminomethane
UTP	Uridinetriphosphate

GENERAL INTRODUCTION

This study was undertaken to investigate the possible role of the digitalis-sensitive (Na^+-K^+)-activated ATPase system in the secretion of fluid and electrolytes and of enzymes by the pancreas. For this purpose we first studied the occurrence of the enzyme system in both dog and rabbit pancreas. Once the presence of the (Na^+-K^+)-activated ATPase system had been established, we determined its properties. Simultaneously we studied the effect of the cardiac glycoside ouabain, which inhibits the enzyme system, on pancreatic fluid and electrolyte secretion in vivo in the dog. The technical limitations of the in vivo approach, however, made it desirable to perform in vitro experiments on the isolated organ. Use of the isolated rabbit pancreas, which is very suited for in vitro studies, because of its very thin sheet-like configuration, enabled us to investigate the characteristics of the secretory system in vitro. These properties were compared to those found for the enzyme system and finally led to the conclusion that active sodium extrusion is the primary and rate-limiting step in exocrine pancreatic fluid and electrolyte secretion.

The secretion of digestive enzymes by the exocrine pancreas was studied first from the point of view of a possible coupling to the (Na^+-K^+)-activated ATPase system. When such a coupling was found to be absent, the role of cyclic AMP as a mediator in enzyme secretion was investigated.

Finally, the morphological aspects of exocrine fluid and electrolyte secretion, and its relation to enzyme secretion, were studied by means of the electron microscope.

The results described in chapters IV through VII have been published in part (Ridderstap, A. S. and S. L. Bonting, 1968a and 1968b; 1969a and 1969b).

MORPHOLOGY AND PHYSIOLOGY OF THE PANCREAS

1. MORPHOLOGICAL, HISTOCHEMICAL AND ELECTRONMICROSCOPIC ASPECTS OF THE PANCREAS

Embryology. The pancreas in mammals appears in the same region and at about the same time as the liver. It is derived from two separate primordia, which as they increase in size, approach each other and fuse generally. The glandular tissue of the organ is formed by budding and rebudding of cords of cells derived from this primordial mass. The terminal parts of the cords gradually take on the characteristic configuration of pancreatic acini, while the more proximal portions form the duct system draining the acini (Patten, 1958).

Ducts. There is considerable variation in the relations of the main pancreatic ducts which persist in the adult. In the dog, for example, there are two ducts: a dorsal one (duct of Santorini) which opens directly into the duodenum and a ventral one (duct of Wirsung) which enters the duodenum via the common bile duct. They represent the two original pancreatic buds which generally appear in mammalian embryos. In other forms the two ducts become confluent within the organ and the terminal part of one duct only is retained. In man the ventral duct persists, while in the rabbit the dorsal duct persists as the definitive pancreatic duct (Patten, 1958).

Localization. The gland of the dog, an example of a compact pancreas, consists of a left part or tail, and a right part or head. The tail part lies along the greater curvature of the stomach and the splenic vessels from the medial margin of the spleen to the back of the pylorus and the hilum of the liver, while the head part starts at this point and lies along the left border of the descending limb of the duodenum. The head of the pancreas extends posteriorly and towards the left, forming thus the uncinate process. As already mentioned, the dog pancreas has two ducts. The duct of Wirsung drains the tail and enters the intestine with or near the common bile duct, while the duct of Santorini on the other hand drains the head and opens 3 to 5 cm below the common bile duct into the duodenum. The organ of the rabbit, an example of a diffuse pancreas, consists of a thin sheet of tissue lying in the mesoduodenum, with one large mass between the portal vein and the vena

cava and another mass in the mesentery of the spleen. There is only one duct, which enters the duodenum about 30 cm below the common bile duct.

Blood supply. The arterial blood supply of the pancreas is mainly derived from the celiac axis and to a lesser degree from the arteria mesenterica superior. The tail of the organ is supplied by several branches from the hepatic and splenic arteries, while the head receives its blood from numerous branches from the superior pancreaticoduodenal artery, which arises from the gastroduodenal artery. Part of the uncinate process is supplied by branches from the inferior pancreaticoduodenal artery, a branch of the arteria mesenterica superior. The blood leaves the organ through numerous veins, which join similar blood vessels deriving from the duodenum to form the pancreaticoduodenal vein, a branch of the portal vein. Other veins from the tail empty directly into the splenic vein and the gastric coronary vein (Eichenberger et al., 1966).

Innervation. The autonomic nervous system plays a very important role in the innervation of the pancreas. The organ is innervated by the vagal and splanchnic nerves respectively (Grossman, 1962).

Histology. The exocrine pancreas is made up of groups of acini which in cross-section show 5 to 8 pyramidal epithelial cells surrounding a small central lumen at the apex of the cell. The acinar cell is elongated and has a broad base and a tapered apex. The cell apex usually contains small highly refractile zymogen granules which, however, are not visible in the hematoxylin and eosin stain after formalin fixation (Herman et al., 1964). Special stains reveal that these secretion granules occupy a major fraction of the apical zone of the cell. In contrast the basal portion of the cell shows considerable basophilia. Nuclei are spherical with abundant chromatin and have one to three nucleoli.

Electron microscopy studies confirmed that the acini are composed of a number of acinar cells and occasional "centroacinar" cells (Ekholm et al., 1962). The acinus is encircled by a thin basement membrane which borders on capillaries and interstitial tissues and also lies in intimate contact with the acinar cell plasma membrane (Robertson, 1961; Sjöstrand and Elfvin, 1962). The apex has microvilli projecting into the central lumen. The main structural components of the cytoplasm of the acinar cells are the filamentous mitochondria, the Golgi apparatus, the zymogen granules and a densely arranged system of membranes that appear in cross-section to be paired (Sjöstrand, 1962).

The duct system of the pancreas may be conveniently divided into three regions. The ductular cells of the smallest lumen which communicates directly with the acinar cell lumen have been called "centroacinar" cells. Preferable is the term "acinoductular", because these cells are in reality ductular cells. The acinoductular cells are continuous

with intercalary ductules which lead into larger ducts called intralobular ductules. The latter extend into interlobular ducts, which connect with the main or accessory ducts (Richards and Fitzgerald, 1962) The ultrastructure of all ductular cells is essentially the same Ekholm et al (1962) showed that acinoductular cells, intercalary and intralobular ductular cells of the exocrine pancreas exhibit marked ultrastructural similarities All three types are characterized by a cytoplasm containing very little endoplasmic reticulum, comparatively few mitochondria, a modest Golgi apparatus and considerable amounts of delicate fibrils. The nucleus is round or oval with marginal indentations The apical surface has microvilli and deviates distinctly with respect to its configuration. In the intralobular ductules this surface very often has blebs which can obtain considerable dimensions These blebs are smaller and fewer in the intercalary ductules and they are but occasionally observed in the acinoductular cells Carbonic anhydrase, an enzyme which catalyses the formation of bicarbonate, was shown by Becker (1962) to be present in the intercalary and intralobular ductular cells The acinar cells, however, contain only very little or no carbonic anhydrase activity.

2 FLUID AND ELECTROLYTE SECRETION

The fluid secreted by the pancreas is isosmotic with plasma and its osmolarity is independent of the rate of flow Therefore, induced alterations in osmotic pressure of plasma are rapidly followed by corresponding alterations in the osmotic pressure of pancreatic juice.

Cationic composition Pancreatic fluid after secretin stimulation has sodium and potassium concentrations which are approximately equal to those in plasma. Both concentrations are independent of flow rate. Johnston and Ball (1930) reported for the dog, sodium and potassium concentrations of 154 (SE 7) and 4 8 (SE 0 9) mmoles per kg H_2O respectively. Dreiling and Janowitz (1956) observed that in human pancreatic juice obtained by duodenal intubation, the concentration of sodium varied between 139 and 143 mmoles per liter and that of potassium between 6 and 9 mmoles per liter. The concentration of calcium, however, was lower (1 7 mmoles/liter, SE 0 3) than in plasma, and magnesium also was present in quite small amounts (0 5 mmoles/liter). Intravenous injection of hypertonic NaCl or KCl solution in the dog (Ball, 1930) increases the concentrations of sodium and potassium in plasma and pancreatic juice to exactly the same degree Intravenously administered radioactive Na^+ appears within 3 min. in the secreted pancreatic juice A maximal concentration of secretory sodium was found within 15 min , with the exception of the early periods the concentration of labelled sodium in the juice closely followed that of serum (Montgomery et al., 1941).

Anionic composition. Anionic composition of the secreted fluid is a function of the rate of flow with the principal anions being bicarbonate and chloride. The concentration of bicarbonate which ranges from 25 to 150 mmoles per liter, varies directly with the flow rate. The chloride concentration on the other hand varies inversely with the bicarbonate concentration; the sum of both concentrations remains relatively constant (154 mmoles/liter, SE. 10). In dog (Hart and Thomas, 1945) and man (Dreiling and Janowitz, 1959) the curve of bicarbonate concentration versus flow rate is a smooth curve, reaching asymptotically a maximum value at high flow rate. Increasing plasma bicarbonate level leads to elevated concentration and output of bicarbonate in pancreatic juice (Rawls et al., 1963). Intravenously injected radioactive CO_2 appears promptly in pancreatic fluid of the dog, with a specific activity 4 to 5 times that of plasma (Ball et al., 1941).

Regulation of secretion. The hormone secretin stimulates flow and bicarbonate secretion without altering the output of enzymes from the gland (Wang et al., 1948). The same effects are obtained with a number of agents, which release secretin from the duodenal mucosa, such as HCl, neutral and acid peptones, soaps, glutamic acid and combination of soap, acid and peptones (Hart and Thomas, 1945). Pancreatic fluid and electrolyte secretion is practically not influenced by the cholinergic nervous system (Grossman, 1962). Brown et al. (1967), however, showed that secretin-stimulated flow in the cat is increased after stimulation of the dorsal vagal trunk. Therefore, in addition to the hormonal regulation, there is also a nervous regulation of fluid and electrolyte secretion by the pancreas.

Cellular origin of secretion. The cellular origin of pancreatic juice production has been a classical problem in pancreatic physiology There is some evidence suggesting that the epithelial cells of the intercalary and intralobular ductules are involved in fluid and electrolyte secretion Ethionine, which causes a destructive lesion in the acinar cells, does not affect the volume of pancreatic juice and its bicarbonate content (Kalser and Grossman, 1954). Alloxan, which gives rise to histological changes in the ductular cell, raises the threshold for secretin stimulation (Grossman and Ivy, 1946) Finally, the high activity of carbonic anhydrase in the ductular cells and its low activity in the acinar cells also favours a role of the ductular cells in fluid and electrolyte secretion (Becker, 1962) It appears, therefore, that the epithelium of the ductules is responsible for exocrine fluid and electrolyte secretion by the pancreas.

Mechanism of secretion. The high, flow-dependent concentration of bicarbonate and the presence of carbonic anhydrase in rather high activity in pancreatic tissue (van Goor, 1948) suggested a role of carbonic anhydrase in the secretion mechanism of pancreatic juice. The decrease in both bicarbonate concentration and flow rate after intra-

venous injection of acetazolamide, a specific carbonic anhydrase inhibitor, appeared to support such a role (Birnbaum and Hollander, 1953; Dreiling and Janowitz, 1959, Pratt and Alkawa, 1962, Rawls et al. 1963, Pak et al., 1966) An active transport of bicarbonate was postulated, assuming a passive movement of the other ions and of water. However, Rawls et al. (1963) showed that an inhibition of more than 99% of pancreatic carbonic anhydrase was required to give a maximal flow inhibition of 50% This strongly suggests that the action of the enzyme cannot be the primary, rate-limiting process in the secretion of pancreatic juice.

3 SECRETION OF DIGESTIVE ENZYMES

With the exception of the lactating mammary gland there is no secretory organ in the body which forms and secretes on demand such large quantities of protein. The pancreas produces a variety of enzymes, which are secreted into pancreatic juice. Practically all proteins secreted are hydrolytic enzymes (Keller and Cohen, 1961, Neurath, 1962; Greene et al., 1963). In the dog, for instance, the following enzymes appear in pancreatic fluid α-amylase, α-lipase, trypsinogen and chymotrypsinogen, procarboxypeptidases A and B and desoxyribonuclease (Marchis-Mouren, 1965). Species differences occur in the relative proportions of these enzymes (Keller et al 1958; Marchis-Mouren et al , 1961). It was demonstrated that the major proteins secreted by the pancreas must be derived from the zymogen granules (Palade et al., 1962). The relative amounts of the enzymes in the zymogen granules and in the secreted juice are identical.

Enzyme synthesis and secretion in pancreas slices incubated in vitro in a physiological saline was demonstrated several years ago for α-amylase (Hokin, 1951a) and for α-lipase and ribonuclease (Schucher and Hokin, 1954) It was found that ten amino acids are required for maximal α-amylase formation in pigeon pancreas slices (Hokin 1951b). All of these amino acids are present in crystalline α-amylase (Caldwell et al., 1954)

Regulation of enzyme secretion. Pancreatic enzyme secretion is subject to hormonal and nervous regulation When pancreozymin, a duodenal hormone released upon passage of food through the intestine, is injected intravenously, an increase in the output of pancreatic enzymes is observed There is, however, no effect on the flow rate (Harper and Raper, 1943). More than 70 years ago Pavlov (1897) demonstrated enzyme secretion by the pancreas after stimulation of the peripheral end of a vagus nerve. He also claimed that there was a slight psychic stimulation of the pancreas. Much later it was shown that the cholinergic nervous system is a strong stimulator of pancreatic enzyme secretion (Tonkich, 1924, Crittenden and Ivy, 1937). Re-

cently Preshaw et al. (1966) presented evidence showing that sham feeding causes a marked increase in pancreatic protein output in dogs. This, of course, is evidence for a psychic stimulation of the pancreas, as proposed by Pavlov (1897). The pancreatic response to sham feeding, as found by Preshaw et al. (1966), was inhibited by acidification of an innervated pouch of the pyloric gland area in a second group of dogs, suggesting that part of this response is mediated by the vagal release of gastrin from the pyloric gland area. The marked inhibitory effect of atropine on enzyme secretion also implies cholinergic control in normal regulation of pancreatic enzyme secretion (Grossman, 1962). Thus with one important exception all the actions of pancreozymin in vivo can be paralleled by vagal stimulation or parasympathomimetic drugs (Kalser and Grossman, 1954). The exception is that the effects of pancreozymin are not blocked by atropine. Experiments with pancreas slices have shown that cholinergic agents or pancreozymin stimulate α-amylase, α-lipase and ribonuclease secretion (Hokin, 1951a; Schucher and Hokin, 1954; Dickman and Morrill, 1957). The action of the cholinergic agents was completely blocked by atropine, while pancreozymin on the other hand was insensitive to atropine. Other experiments in vitro also demonstrated that pancreozymin stimulates the transfer of digestive enzymes across the acinar cell membrane, and not intracellular enzyme synthesis (Dickman and Morrill, 1957).

Cellular site of enzyme secretion. The classical histological studies of Heidenhain (1875) and Langley (1879) demonstrated the presence of zymogen granules in the acinar cells of the pancreas and suggested that they are the storage sites of the digestive enzymes. Isolation of these granules by differential centrifugation revealed that the zymogen granule fraction contains the highest specific activity of α-amylase, α-lipase and protease of any of the fractions isolated (Hokin, 1955). At the present time it is generally accepted that the digestive enzymes are synthetized by the acinar cells and stored in the zymogen granules before release.

Intracellular transport and storage. Four stages along the intracellular pathway followed by the exportable enzymes from their site of synthesis to their site of discharge can be identified (Fig. 1). There is also some knowledge about the order in which these stages follow each other, and concerning the approximate time required to move from one stage to the next along this pathway. The first stage (ribosomal) marks the end of the synthetic phase and the beginning of the transport of exportable enzymes within the acinar cell (Siekevitz and Palade, 1960a and 1960b). The second stage (intracisternal) follows the segregation of the exportable proteins in the cavities of the rough-surfaced cisternae of the endoplasmic reticulum and may involve an intracisternal granule (Palade, 1956; Siekevitz and Palade, 1958 and 1960a). The third stage is located in the Golgi region and involves the progressive (Fig.

1, 3 to 3′) concentration of the secretion product in a smooth-surfaced vacuole (Caro, 1961). The fourth stage corresponds to the storage of the concentrated product in zymogen granules. Stage 4′ marks the discharge of such a granule into the acinar lumen (Palade, 1959).

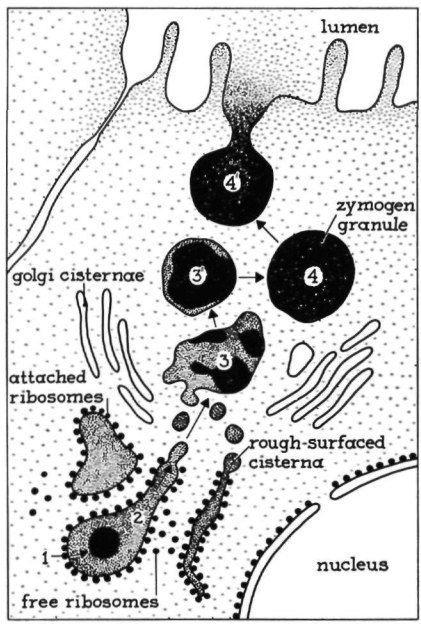


Fig. 1. Intracellular transport, storage and secretion of exportable proteins by the pancreas (after Palade et al., 1962).

Mechanism of enzyme secretion. Induction of enzyme secretion is usually accompanied by a rapid turnover of labelled phosphate in phosphatidic acid and phosphoinositide of the microsomal fraction of the gland cells (Hokin and Hokin, 1965). The relation of turnover in specific phospholipids to the process of protein secretion is not yet clear. The zymogen granules which harbour the exportable proteins show little turnover of phospholipid. Using pigeon pancreas slices it was recently found that calcium ions play a role in induction of enzyme secretion (Hokin, 1966). However, in the absence of calcium ions, acetylcholine does not induce α-amylase secretion in vitro but still causes the turnover of phospholipids. This suggests that the effect on phospholipid turnover is not directly related to the transport of protein across the cell membrane of the acinar cell, but that it may be the result of increased membrane synthesis following zymogen granule release.

ACTIVE CATION TRANSPORT AND ITS ENZYMATIC BASIS

1 MECHANISM OF ACTIVE CATION TRANSPORT

Experiments carried out on cells of a variety of tissues have demonstrated that Na^+ is transported from the cytoplasm to the extracellular fluid against an electrochemical gradient (Ussing, 1960). Transport against an electrochemical gradient is called active transport, because it requires energy. Experiments on nerve (Caldwell, 1956, Caldwell and Keynes, 1957) and on erythrocytes (Dunham, 1957) have demonstrated that the energy for cation transport is derived from ATP.

Studies on erythrocytes have shown that active sodium transport depends on the concentration of K^+ in the extracellular fluid and that there seems to be a coupling between the outward Na^+ transport and inward transport of K^+ (Glynn 1956) Another characteristic of active Na^+ transport is that it is inhibited by cardiac glycosides (Schatzmann, 1953, Glynn, 1957).

From these experiments it became clear that the Na^+ transport system should be localized in the cell membrane and have an affinity for Na^+ that is higher than for K^+ on the inside of the cell, while the affinity for K^+ is higher on the outside of the cell membrane. Furthermore the system must be capable of hydrolyzing ATP at a rate dependent on the intracellular sodium concentration and extracellular potassium concentration respectively.

The search for this transport system finally led to the discovery of an (Na^+-K^+)-activated, ATP-hydrolyzing enzyme system by Skou (1957) in crab nerve. This enzyme meets the requirements mentioned above. There is a close correlation between the effect of cardiac glycosides on cation transport in the intact cell and the (Na^+-K^+)-activated ATPase system in erythrocytes (Post et al., 1960, Dunham and Glynn, 1961) The erythrocyte enzyme system even demonstrates the same half-activation concentration for Na^+ and K^+ as the transport system in the erythrocyte ghosts

2 OCCURRENCE OF THE ENZYME SYSTEM

Occurrence. The ouabain-sensitive (Na^+-K^+)-activated ATPase system has been demonstrated in a large number of tissues in the past

few years. The most extensive studies on the occurrence of the enzyme system were made by Bonting et al. (1961). They found the enzyme system in 29 of 36 tissues from the cat The highest activity was found in nervous tissue and in tissue concerned with secretory functions. It was undetectable in tissues without cells or with a very low cell density. The pancreas was not studied. Bonting et al. (1962) also studied the occurrence of the $(Na^{-}\text{-}K^{+})$-activated ATPase system in the tissues in which an active Na^{+} and K^{+} transport sensitive to cardiac glycosides was known. Investigating 21 tissues from 10 different species, they found the $(Na^{+}\text{-}K^{+})$-activated ATPase system present in significant quantities in all tissues

Localization The distribution of the enzyme system in centrifugal fractions of brain and liver indicated its presence in the cell membrane (Bonting et al., 1962). Localization of the enzyme system in the cell membrane was clearly proved in the case of the erythrocyte (Glynn, 1962; Hoffman, 1962a, Whittam, 1962). Bonting and Caravaggio (1962) found the enzyme system localized in the sheath of the squid axon, and Cummins and Hyden (1962) showed that in the neuron the enzyme system is localized in the membrane part

3 PROPERTIES OF THE ENZYME SYSTEM

The enzyme system hydrolyzes ATP to ADP and P_i. It differs from other ATP-hydrolyzing enzymes in that it requires, in addition to Mg^{++}, both Na^{+} and K^{+} in the substrate medium for activity (Skou, 1957).

Affinity for cations. Omission of either Na^{+} or K^{+} from the substrate medium leads to a lower ATPase activity, which represents a separate entity, the Mg^{++}-stimulated ATPase system. If the substrate medium contains Na^{+} as well as Mg^{++} the addition of one of the cations K^{+}, Rb^{+}, Cs^{+}, NH_4^{+} or Li^{+} gives rise to a considerable increase in enzyme activity (Skou, 1957 and 1960). In crab nerve in the presence of Na^{+} and Mg^{++} the affinity decreases in the order: K^{+}, Rb^{+}, NH_4^{+}, Cs^{+}, Li^{+} (Skou, 1957). With K^{+} and Mg^{++} present in the substrate medium increasing concentrations of Na^{+} also increase the enzyme activity considerably and to the same level (Bonting et al, 1964b).

The half-maximal activation concentration for K^{+} in the presence of Na^{+} is 1 6 mM K^{+} for crab nerve enzyme (Skou, 1957), whereas it is 1 0 mM K^{+} for rabbit brain enzyme, 0.5 mM K^{+} for rabbit kidney enzyme (Skou, 1962), and 0 5 mM K^{+} for rabbit lens (Bonting et al., 1963) The half-maximal activation concentration for Na^{+} in the presence of K^{+} and Mg^{++} is 12 5 mM Na^{+} for herring gull salt gland (Bonting et al, 1964b), 13.5 and 11.7 mM Na^{-} for the rectal gland of spiny dog fish and sand shark respectively (Bonting, 1966).

A kinetic analysis of the effect of Na^{+} and of K^{+} on the enzyme

system activity suggests that it has two sites with affinities for cations (Skou, 1960), one site where the affinity for Na^+ is 6 to 8 times higher than that for K^+ and where K^+ by competition can replace Na^+, and a second site with high affinity for K^+ and a very low affinity for Na^+. For maximal activity the enzyme requires Na^+ at the first site and K^+ at the second At concentrations of K^+ that are high compared to the Na^+ concentration, K^- displaces Na^+ from the first site by competition and thus decreases the activity.

The activity of the (Na^+-K^+)-activated ATPase system is strongly inhibited by Ca^{++} in low concentrations (Skou, 1957; Somogyi, 1964) This inhibition can to some extent be reversed by an increase in the Na^+ concentration (Portius and Repke, 1963).

Effect of cardiac glycosides. Cardiac glycosides inhibit the (Na^+-K^+)-stimulated ATPase activity, but not that of the Mg^{++}-stimulated ATPase This has been observed for the enzyme system isolated from all the tissues mentioned hitherto. The sensitivity to cardiac glycosides, however, differs for the enzyme system from different tissues of the same animal and from the same tissue of different animals (Bonting et al , 1962). This is not surprising since it is known that the sensitivity of different animals to cardiotonic steroids varies within wide limits (Lendle and Mercker, 1961).

Bonting et al. (1964a) found that Erythrophleum alkaloids, erythrophleine and cassaine, are also capable of inhibiting the enzyme system. As in the case of cardiac glycosides, they are unable to inhibit the Mg^{++}-stimulated ATPase activity In several tissues of different species it was demonstrated that erythrophleine is a more potent inhibitor of the (Na^+-K^+)-activated ATPase activity than ouabain (Bonting et al , 1964a; Vates et al., 1964; Bakkeren and Bonting 1968a).

The inhibition of the (Na^+-K^+)-stimulated ATPase activity by ouabain is antagonized by increasing the K^+ concentration (Dunham and Glynn, 1961, Portius and Repke, 1962). This effect suggests that cardiac glycosides inhibit the enzyme system by a displacement of K^+ from the K^+ site Another possibility is that cardiac glycosides interfere with the coupling between Na^+ and K^+ (Askari and Fratantoni, 1963)

Cardiac glycosides in very low concentrations on the other hand stimulate the enzyme activity in a variety of tissues from different species (Repke, 1963; Lee and Yu, 1963; Bonting et al , 1964b; Palmer and Nechay, 1964, McClane, 1965; Bonting, 1966; Nahmod and Walser, 1966, Oppelt and Palmer, 1966). Erythrophleine also shows this effect (Bonting et al., 1964a).

pH dependence. The pH optimum for the enzyme system from several vertebrate tissues has been found to range from 7.0 to 7.3 (Bonting, 1966; Bakkeren and Bonting, 1968a), while the pH optimum of the Mg^{++}-activated ATPase system ranges between 8 4 and 9 2 (Bonting et al , 1963; Ridderstap and Bonting 1969a).

4. COMPARISON OF PROPERTIES OF ENZYME SYSTEM AND ACTIVE TRANSPORT SYSTEM

The substrate for the (Na^+-K^+)-activated ATPase system is ATP; with ITP, GTP or UTP as substrate, there is little or no activation by Na^+ and K^+ (Post et al., 1960; Skou, 1960). This is in complete agreement with the findings of Hoffman (1962a) that active Na^+ transport in the erythrocyte ghost requires ATP as an energy source, while with ITP there is only a very slight transport of sodium and with GTP and UTP transport is completely abolished.

The half-maximal activation concentrations for Na^+ and K^+ transport in erythrocytes correspond to the Na^+ and K^+ concentrations that cause half-maximal activation of the (Na^+-K^+)-activated ATPase system of erythrocyte membranes (Post et al., 1960). There is also a correlation between the enzyme activity and the intracellular Na^+ and K^+ concentrations. The erythrocytes of one type of sheep have a low Na^+ and a high K^+ concentration; another type of sheep have a high Na^+ and a low K^+ concentration. The (Na^+-K^+)-stimulated ATPase activity of erythrocytes with the high K^+ concentration is about four times higher than that from the red blood cells with the low K^+ concentration (Tosteson et al., 1960; Tosteson, 1963). Human erythrocytes contain 20 mmoles per liter of Na^+ and 100 mmoles per liter of K^+, whereas cat erythrocytes contain 104 mmoles per liter of Na^+ and 6 mmoles per liter of K^+. The enzyme activity from human erythrocytes is approximately 8 times higher than that from cat erythrocytes (Bonting et al., 1961). Both the (Na^+-K^+)-activated ATPase system and active Na^+ transport in erythrocyte ghosts are inhibited by Ca^{++} (Hoffman and Ryan, 1960). In the latter case the Ca^{++} had to be present in the inside solution. In six different tissues Bonting and Caravaggio (1963) have demonstrated a significant correlation between the activity of the enzyme system and active cation transport over a 25,000 fold range. The ratio of cation transported to ATP hydrolyzed was nearly 3.

Cardiac glycosides in low concentrations are specific inhibitors of active cation transport (Schatzmann, 1953; Glynn, 1957). Cardiotonic steroids are also potent inhibitors of the (Na^+-K^+)-activated ATPase system from all sources. The ouabain concentration that causes half-maximal inhibition of the active transport in the intact human erythrocyte is 3.7×10^{-7} M (Gill and Solomon, 1959), while half-maximal inhibition of the enzyme system occurs at 10^{-7} (Post et al., 1960) to 1.3×10^{-7} M ouabain (Dunham and Glynn, 1961). There is a correlation between the inhibitory effects of different cardiac glycosides on the transport system and the (Na^+-K^+)-activated ATPase system of erythrocytes (Dunham and Glynn, 1961). The inhibition by cardiac glycosides of both the active transport and enzyme system is antagonized by K^+ (Glynn, 1957; Hoffman, 1962a; Bonting et al., 1963).

These findings clearly show that the (Na^+-K^+)-activated ATPase

system is closely related to or identical with the cation pump in several types of cells

5 MECHANISM OF THE CATION PUMP

It has been shown that the Na^+ and K^+ stimulated hydrolysis of ATP catalyzed by the (Na^+-K^+)-activated ATPase system involves at least two separate steps. The results of the ATP-P_i exchange reaction (Skou, 1960) and experiments with radioactive ATP (Post and Rosenthal, 1962, Charnock et al , 1963; Fahn et al , 1963, Heinz and Hoffman, 1963; Rose, 1963) suggest that the first step is a transfer of an energy-rich phosphate bond to a compound in the system, requiring the presence of Mg^{++} and Na^+ ions. The second step is a dephosphorylation of the phosphorylated intermediate in the presence of K^+ ions, this step is sensitive to cardiac glycosides (Post and Rosenthal, 1962, Albers et al , 1963). The dephosphorylation step in the Na^+-K^+ ATPase reaction has been correlated with a K^+-activated phosphatase system present in a variety of tissues (Ahmed and Judah 1964, Albers and Koval, 1966, Nagai et al , 1966).

Based on our present knowledge concerning the relation between the (Na^+-K^+)-activated ATPase system and the cation pump one can say that the initial reaction step consists of the rapid reversible formation of a phosphorylated complex, and that this first step is accompanied by an outward transport of Na^+ ions. The formation of the phosphorylated compound is followed by an almost irreversible decomposition to P_i and free enzyme, and an inward transport of K^+ ions (Glynn 1968).

Two molecular models have been proposed for the Na^+-K^+ ATPase system, based on its known properties. The first is from Opit and Charnock (1965), who assume that the (Na^+-K^+)-activated ATPase system is localized at the interior side of the cell membrane, this protein layer would contain a large number of negatively charged groups, able to bind Na^+ and K^+ ions A change in the Na^+/K^+ ratio inside the cell would give rise to a redistribution of electron density in the protein chain In the presence of a great number of Na^+ ions phosphorylation would take place accompanied by a rotation of a major part of the negatively charged groups towards the exterior of the cell. At the outside a marked preference would now be exhibited for K^+ by the cation binding groups, and thus the Na^+ ions at these sites would be exchanged for K^+. Hence Na^+ has been transported from the intracellular to the extracellular environment. The adsorption of K^+ again would cause a redistribution of the electron density, leading to liberation of phosphate from the phosphoprotein and allowing the molecule to return to its initial shape As a result the cationic binding sites with their adsorbed K^+ ions would rotate towards the cell interior Within

the cell some of these adsorbed K^+ ions are now displaced by Na^+ and the cycle of reaction starts again.

Albers and associates (Fahn et al., 1966; Albers, 1967; Albers et al., 1968) have proposed a model for the Na^+-K^+ ATPase reaction, that resembles that of Opit and Charnock in many ways. They postulated the existence of two forms of the enzyme system: the "cis"-form with cation binding sites located at the side of the phosphorylation site (intracellular), and the "trans"-form with binding sites at the opposite side (extracellular). The "cis"-form would have a very high affinity for Na^+ ions, and the "trans"-form for K^+ ions. By means of the phosphorylation reaction the "cis"-form would be transferred to the "trans"-form and thus Na^+ ions would be transported to the outside. Dephosphorylation, on the contrary, would change the "trans"-form into the "cis"-form and in this way K^+ ions would be transported to the inside of the cell, with orthophosphate being released at the inside of the membrane

6 PHYSIOLOGICAL FUNCTION OF THE CATION PUMP

Bonting et al. (1961), in studying the occurrence of the (Na^+-K^+)-activated ATPase, found the enzyme system to be present in 29 out of 36 tissues of the cat The highest activities were noted in nervous and secretory tissues, including the ciliary body and the choroid plexus. The suggestion was made that the enzyme system might be active in the re-establishment of membrane potentials after depolarization, and in the secretion of certain fluids, notably aqueous humour and cerebrospinal fluid. In individual cells the cation pump is responsible for the unequal distribution of Na^+ and K^+ ions between intracellular and extracellular environment. The high intracellular K^+ concentration is essential, because several enzymatic processes within the cell, such as protein synthesis and formation of acetyl-coenzyme A, are stimulated by K^+ ions (Kernan, 1965).

In the past evidence has been presented showing that the sodium pump, which is identical with the (Na^+-K^+)-activated ATPase system, plays an essential role in ion transport in lens (Bonting et al., 1963) and leukocytes (Block and Bonting, 1964) and in Na^+ reabsorption in the toad bladder (Bonting and Canady, 1964). More recently the Na^+ pump has been demonstrated to play a part in cation transport in Escherichia coli (Hafkenscheid and Bonting, 1968), in liver (Bakkeren and Bonting, 1968b), and in cochlear function (Kuijpers et al., 1967). The Na^+ pump has also been established as the primary and rate-limiting process in the formation of aqueous humour (Simon et al., 1962; Bonting and Becker, 1964) and cerebrospinal fluid (Vates et al., 1964), and in sweat secretion (Slegers, 1968).

In cases of fluid secretion the secretory cells possess a considerable

(Na^+-K^+)-stimulated ATPase activity, which acts as a Na^+ pump. In analogy to the "local osmosis" proven by Diamond (1962) for the gall bladder, it would appear that this pump system secretes Na^+ from the cytoplasm into a restricted space in or just beyond the cell membrane and thus causes locally a high concentration of Na^+. In response to this local osmotic gradient, water crosses the membrane, and so passively follows the actively extruded Na^+.

Finally it must be stated here that the active transport of amino acids, sugars and other substances has in many cases been shown to be coupled to the Na^+ pump (Csáky, 1963a and 1963b; Bittner and Heinz, 1963; Fox et al., 1964; Vidaver, 1964; Curran, 1965).

From the properties of the (Na^+-K^+)-activated ATPase system described here it seems a reasonable assumption that it might also play a role in pancreatic fluid and electrolyte secretion, and possibly also in pancreatic enzyme secretion. The primary purpose of our study was to investigate whether such a role exists.

MATERIALS AND METHODS

1. MATERIALS

The following chemicals and materials were used: acetazolamide (6); acetylcholine chloride (4); adenosine 3′, 5′-monophosphate, sodium salt (1); adenosinetriphosphate, disodium salt (7); Amberlite IR-120 (H^+-form) (3); digoxin (4); Epon 812 (12); erythrophleine sulphate (4); ethylenediaminetetraacetate, disodium salt (4); glutaraldehyde (4); halothane (9); heparin (10); hexahydroscillaren A (7); lactic acid (3); ouabain (strophanthine-g) (4,5); pancreozymin (2); scillaren A (7); secretin (2); sodium thiopentone (2); starch (4); theophylline (4); tris-(hydroxymethyl)-aminomethane (4,8); Topostasin (11).

1. C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.
2. Boots Pure Drug Co. Ltd., Nottingham, England.
3. The British Drug Houses Ltd., Poole, England.
4. E. Merck A.G., Darmstadt, Germany.
5. Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.
6. American Cyanamid Company, Pearl River, New York, U.S.A.
7. Sandoz A.G., Basel, Switzerland.
8. Sigma Chemical Company, St. Louis, Missouri, U.S.A.
9. Imperial Chemical Industries Ltd., Macclesfield, England.
10. N.V. Organon, Oss, The Netherlands.
11. S. Hoffmann-La Roche & Co. A.G., Basel, Switzerland.
12. Shell Nederland Chemicaliën N.V., The Hague, The Netherlands.

2. METHODS

a. *Preparation of the Tris salt of ATP*

Since the sodium salt of ATP cannot be used in the sodium-free substrate medium in the ATPase assay and the potassium salt was commercially not available, the sodium salt was converted into the tris salt. For this purpose 500 mg of ATP (disodium salt) dissolved in 3 ml of water was applied to an Amberlite IR-120 (tris-form) column, containing 70 ml of the ion-exchanger. The nucleotide was eluted by means of distilled water. Use of a fractioncollector (LKB, Stockholm), provided with a UV-absorptionmeter (Uvicord, LKB, Stockholm)

enabled us to collect the eluate in 10-min. fractions. All fractions belonging to the peak formed in the absorption-curve were pooled and lyophilized and stored at -20° C.

b. *Tissue preparation*

Animals (dogs and rabbits) were electrocuted and exsanguinated from the carotic arteries. The dog pancreas was excised immediately and the wet weight of the gland was measured. Aqueous homogenates, usually 10% (w/v), were prepared at 0° to 4° C in Potter-Elvehjem ground-glass tissue grinders and 0.5 ml aliquots of the homogenate were shell-frozen in 13 x 100 mm tubes and lyophilized at -20° C and stored at -25° C until assayed. In the case of the rabbit the excised organ was first liberated as far as possible from the surrounding fat and mesentery. The dissected pancreas pieces were then frozen on dry ice, lyophilized and stored at -25° C until assayed.

c. *Urea pretreatment*

Since the relative (Na^+-K^+)-stimulated ATPase activity in rabbit pancreas was rather low and the Na^+-K^+ ATPase must be determined by a differential assay, difficulties were encountered in studying the properties of the enzyme system (chapter VI). Therefore a pretreatment of the enzyme preparation with concentrated urea solution was performed. Pretreatment with urea was carried out by incubating the homogenate for 15 min. at 0° C in the presence of 1.5 M urea. The homogenate was then centrifuged in a preparative ultracentrifuge (Spinco L2-50, Beckman) for 60 min. at 100,000 x g and the supernatant discarded to remove practically all urea. The required amount of water was added to the precipitate and after another 30-min. period at 0° C the suspension was used for the ATPase assay.

d. *ATPase assays*

In order to determine the (Na^+-K^+)-stimulated ATPase activity use was made of the following characteristics of the enzyme system, viz. activation by sodium and potassium ions and inhibition by ouabain. Both Mg^{++}-activated and (Na^+-K^+)-stimulated ATPase activities were determined by means of a modification of the method described by Bonting et al. (1961) with the substrate media listed in Table 1. Total ATPase activity was measured in the substrate medium A which gives complete activation of the (Na^+-K^+)-activated ATPase system. Mg^{++}-stimulated ATPase activity was determined in four inhibitory media, wherein the (Na^+-K^+)-stimulated ATPase activity was inhibited either by omission of Na^+ or K^+ or by addition of ouabain.

The lyophilized enzyme preparations were suspended in twice distilled water to give final concentrations of 7-8 mg original wet weight per ml for dog pancreas and 9-10 mg dry weight per ml for rabbit pan-

creas. Aliquots of 0.25 ml of the suspension were mixed with 3.75 ml of each of the following substrate media: medium A (complete), medium B (no K^+), medium C (no Na^+), medium D (10^{-4} M ouabain added) and medium E (no K^-, 10^{-4} M ouabain added). From each mixture six aliquots of 0.5 ml were placed in test tubes, three of which were incubated for 60 min. at 37° C; the others remained in ice and received trichloroacetic acid immediately. To all tubes 2.25 ml of a

Table 1

CHEMICAL COMPOSITION OF THE VARIOUS SUBSTRATE MEDIA *

Medium	A	B	C	D	E
ATP **	2	2	2	2	2
Mg^{++}	1	1	1	1	1
K^+	5	—	5	5	—
Na^-	58	63	—	57	62
EDTA	0.1	0.1	0.1	0.1	0.1
Tris-buffer pH 7.5	92	92	151	91	91
Ouabain	—	—	—	0.1	0.1

* All concentrations are expressed in mmoles/liter; only other ionic species Cl^-.
** In media A, B, D and E ATP (disodium salt) was used; in medium C ATP (Tris salt) was used.

10% (w/v) trichloroacetic acid was added and after mixing and centrifugation 1.5 ml aliquots of the supernatant were transferred to other test tubes containing 1.5 ml of a colour reagent. The colour reagent was prepared by dissolving 400 mg $FeSO_4$ in 5 ml of a 1% NH_4-molybdate solution in 1.15 N H_2SO_4 and was used within 2 hours after its preparation. The resulting colour was read within 2 hours at 700 mμ in a Zeiss spectrophotometer (PMQ II).

Phosphate standards were prepared in triplicate from mixtures containing 0.2 ml 50 mM KH_2PO_4 + 20 ml medium A and 0.2 ml 50 mM KH_2PO_4 + 10 ml medium A respectively; the first standard contained 50 μmol P_i, the second contained 25 μmol P_i. Reagent blanks and inorganic phosphate standards were included in each experiment and served to convert the optical density into mmoles of inorganic phosphate released per gram dry weight of tissue per hour incubation at 37° C, after correction with the readings obtained for the unincubated tubes. Enzyme activity was expressed as moles ATP hydrolyzed per kg dry weight per hr at 37° C.

The Mg^{++}-activation curve was determined by varying the Mg^{++} concentration in media A and E (K^+-free, 10^{-4} M ouabain) from 0 to 6 mmoles per liter, while the ATP concentration was kept constant at 2 mmoles per liter. The K^+-activation curve was obtained by adding KCl to medium B (K^+-free) in final concentrations of 0 to 36 mM K^+ for dog pancreas and of 0 to 18 mM K^+ for rabbit pancreas. The Na^+-activation curve was determined by adding NaCl to medium C (Na^+-free) to give final concentrations between 0 and 100 mM Na^+ for dog pancreas and between 0 and 80 mM Na^+ for rabbit pancreas. The ouabain inhibition curve was determined by adding ouabain (10^{-10} to 10^{-2} M final concentration) to medium A (5 mM K^+), both for dog and rabbit pancreas. A similar procedure was followed in determining the erythrophleine inhibition curve for rabbit pancreas. The pH-activity curve was obtained by preparing media A and E (no K^+, 10^{-4} M ouabain) with Tris-HCl and Tris-histidine buffers in a pH range from 6.2 to 9.8. The pH of each resulting medium was measured and used in plotting the assay results.

e. *Chemical composition of the bathing solution*

In order to study pancreatic secretion in vitro the rabbit pancreas was isolated and subsequently incubated in a balanced salt solution. The exact chemical composition of this solution is given in Table 2. The freshly prepared solution was always kept in a glass stoppered vessel in the cold, and was used the same day for one or more experiments.

Table 2

COMPOSITION OF THE BATHING FLUID

	Normal solution	Low Na^+ solution
Na^+ *	170	25
K^+	4.9	10
Ca^{++}	2.5	2.5
Mg^{++}	1.2	1.2
HCO_3^-	25	25
$H_2PO_4^-$	1.2	1.2
pH	7.2	7.2
glucose	5.5	5.5
sucrose	—	140

* All concentrations are expressed in mmoles/liter; only other ionic species Cl^-.

In the experiments in which the effect of a low sodium environment on exocrine pancreatic secretion was investigated the solution was modified in the following way: the sodium concentration was lowered from 170 to 25 mmoles per liter. Sucrose was added to the modified solution in order to maintain osmolarity.

f. *Electrolyte determinations*

Sodium and potassium determinations were carried out in diluted samples of pancreatic secretion. Twenty µl of pancreatic juice were diluted with twice distilled water to a final volume of 4 0 ml. This volume was sufficient for flame photometric measurements of both sodium and potassium. An Eppendorf flame photometer was used in these determinations. Solutions containing 0.8 mM Na^+ + 0.08 mM K^+ and 0.2 mM Na^+ + 0 02 mM K^+ respectively were used as standards The sodium and potassium curves were linear between 1 x 10^{-4} and 8 x 10^{-4} M Na^+ and 1 x 10^{-5} and 8 x 10^{-5} M K^+ respectively. All determinations were carried out in duplicate.

Total CO_2 was determined manometrically in a Natelson microgasometer. Thirty µl of diluted samples (dilution factor 10) were introduced together with the same volume of a 1 N lactic acid solution into the microgasometer. Carbon dioxide was released from the aqueous solution by shaking under reduced pressure for 1 min. and brought to a constant volume to record manometer reading P_1. Simultaneously the temperature was noted. Then the CO_2 was absorbed in 30 µl of a 3 N NaOH solution and the pressure P_2 of the residual gasses was measured at the same volume. The pressure drop (P_1-P_2) expressed in mm Hg was multiplied by a factor supplied by the manufacturer for each temperature at the time of measurement and by the dilution factor to give the molar CO_2 concentration. All determinations were carried out in duplicate.

Chloride was determined coulometrically. To one ml of diluted samples (dilution factor 50) 2 ml of a nitricacid (0.1 N)-aceticacid (10%) reagent and two drops of a 0.62% gelatin solution were added and the chloride content was measured in an Aminco-Cotlove titrator. Duplicate standard and blanks were titrated prior to the titration of the samples. From the titration results obtained for the standard which were corrected for the blanks, a calibration factor K was calculated (equation (a)).

$$K = \frac{\text{ml of standard x concn. of standard}}{\text{average net seconds of the standard}} \qquad \text{(a)}$$

The concentration of chloride in the samples was calculated according to equation (b).

$$[Cl^-] = \frac{K \times \text{net seconds of sample}}{\text{ml of sample}} \qquad (b)$$

Multiplication by the dilution factor gave the chloride concentration in pancreatic juice.

g. *α-Amylase determination*

Pancreatic α-amylase activity was measured by determination of reducing groups from starch after a 3 min. incubation period with enzyme at 30° C (Bernfeld, 1955). One ml of properly diluted pancreatic juice was incubated for 3 min. at 30° C with one ml of a 1% starch solution (w/v). The enzyme reaction was interrupted by the addition of 2 ml of a 1% dinitrosalicylic reagent. The tube containing this mixture was heated for 5 min. in boiling water and then cooled in running tap water. After addition of 20 ml of water, the optical density of the solution was determined at 570 mμ in a Zeiss spectrophotometer (PMQ II). Blanks were prepared in the same manner with water. A calibration curve established with maltose was used to convert the readings, corrected for the blanks into mg of maltose. α-Amylase activity was expressed in terms of mg of maltose liberated in 3 min at 30° C by one ml of the enzyme containing solution. All determinations were carried out in duplicate.

h. *Protein determination*

Pancreatic protein was determined according to the method of Lowry et al. (1951). To 0 2 ml properly diluted samples one ml of an alkaline copper (a mixture containing 50 ml of a 2% Na_2CO_3 in 0 10 N NaOH and 1 ml of a 0.5% $CuSO_4$. 5 H_2O in 1% Na or K tartrate) solution was added and the solution was mixed. After approximately 10 min. 0.10 ml of a diluted Folin-Ciocalteu phenol reagent was added and mixed rapidly. After 30 min. or longer, optical density was read at 750 mμ in a Zeiss spectrophotometer (PMQ II). A calibration curve established with bovine albumin was used to convert the readings into mg of protein. All determinations were carried out in duplicate.

OCCURRENCE OF AN (Na^+-K^+)-ACTIVATED ATPase SYSTEM IN DOG PANCREAS

INTRODUCTION

In an attempt to elucidate the possible role of the (Na^+-K^+)-activated ATPase system in exocrine pancreatic fluid and electrolyte secretion, our first subject of study has been to investigate the occurrence of the enzyme system in homogenates of dog pancreas. After its presence had been established we have determined its properties. In this chapter the results obtained in this study are reported and discussed.

1 ACTIVITIES OF ATPase

Both Mg^{++}-stimulated ATPase and (Na^+-K^+)-activated ATPase activities were determined by means of a modification of the method and substrate media described by Bonting and associates (1961) A detailed description of this method is found in the third chapter The total ATPase activity was calculated from the activity in the complete medium A, while the average activity in the inhibitory media B (no K^+), C (no Na^+), D (10^{-4} M ouabain) and E (no K^+, 10^{-4} M ouabain) was

Table 3

EFFECTS OF SUBSTRATE MEDIA ON DOG PANCREAS ATPase ACTIVITY

Medium	%
A (complete)	100
B (no K^+)	84 1 + 2 5
C (no Na^+)	62 6 ± 2 8
D (10^{-4} M ouabain)	74 9 ± 1 2
E (no K^+, 10^{-4} M ouabain)	73 0 ± 2 0
Average B-C-D-E	73 6 + 6 4

ATPase activity in medium A (total ATPase activity) set at 100, data for media B, C, D and E (means with standard errors from three determinations) indicate the activity remaining after inhibition of the (Na^+-K^+)-stimulated ATPase activity.

considered to represent the Mg^{++}-stimulated ATPase activity The difference between the total ATPase activity found in the complete medium and the Mg^{++} ATPase activity, calculated from the inhibitory media was considered to represent the Na^{+}-K^{+} ATPase activity

The relative ATPase activities in the various substrate media for dog pancreas are presented in Table 3. There was an average decrease of 26% in ATPase activity upon omission of sodium and potassium or addition of 10^{-4} M ouabain, which must represent the ouabain-sensitive (Na^{+}-K^{+})-activated ATPase activity. The inhibition in the potassium-free medium was approximately 10% less than the average inhibition for all media This is probably due to partial activation of the (Na^{+}-K^{+})-activated ATPase system by small amounts of tissue potassium present in the incubation mixture (approximately 0 25 mmoles/liter K^{-}), since the half-maximal activation concentration of potassium is only 0 6 mM (Fig. 3). The inhibition in the sodium-free medium on the other hand was significantly higher than in the other media If we assume that full inhibition of the Na^{+}-K^{+} ATPase activity was obtained in media D and E, this would indicate that the Mg^{++}-stimulated ATPase is somewhat sodium-sensitive The reduced inhibition in the potassium-free medium and the increased inhibition in the sodium-free medium have previously been reported for rabbit brain and kidney, rabbit ciliary body (Bonting et al., 1964a) cat choroid plexus (Vates et al., 1964), salt gland of the herring gull (Bonting et al., 1964b), rectal gland of

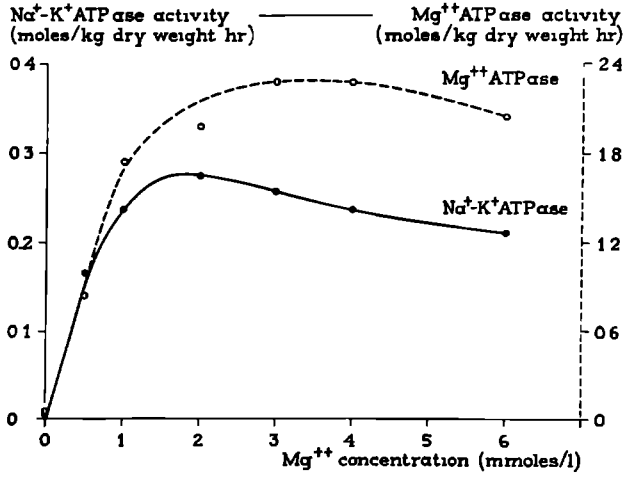


Fig 2 Effect of Mg^{++} concentration on the activities of Mg^{++} ATPase (o - - - - o) and Na^{+}-K^{+} ATPase (● – ●) in dog pancreas homogenates Enzyme activities were measured in media A and E (each containing 2mM ATP), in which the Mg^{++} concentration was varied from 0 to 6 mM The Mg^{++} ATPase activity was calculated from the activity in medium E and the Na^{+}-K^{+} ATPase activity from the difference between the activities in media A and E

elasmobranchs (Bonting, 1966) and rat liver (Bakkeren and Bonting, 1968a). The best values for the (Na^+-K^+)-stimulated ATPase activity could, therefore, be obtained as the difference between activities in media A and D.

The absolute activity for Na^+-K^+ ATPase in dog pancreas was 0.33 (SE: 0.010) and for Mg^{++} ATPase 1.14 (SE 0.183) moles ATP hydrolyzed per kg dry weight of tissue per hour at 37° C.

2. PROPERTIES OF THE ENZYME SYSTEM

The (Na^+-K^+)-activated ATPase system is characterized by the fact that, besides magnesium ions, the enzyme system needs both sodium and potassium ions for its activation, while it is inhibited by cardiac glycosides like ouabain, digoxin and scillaren A and by Erythrophleum alkaloids, such as erythrophleine and cassaine in low concentrations. We have investigated these properties for the Na^+-K^+ ATPase system in dog pancreas, while we also determined the pH optimum of the system.

The effect of increasing the Mg^{++} concentration from 0 to 6 mM, while the ATP-level was kept constant at 2 mM, is shown in Fig. 2. In the absence of Mg^{++} there was virtually no ATPase activity. Mg^{++} ATPase activity was maximal at 3 to 4 mM Mg^{++}, approximately 18% higher than at the routinely used level of 1 mM Mg^{++}. Na^+-K^+ ATPase activity was maximal at 2 mM Mg^{++}, about 16% higher than at 1 mM Mg^{++}, and began to decrease at 6 mM Mg^{++}. So the optimal ratio Mg^{++}/ATP for the Na^+-K^+ ATPase system was 1.

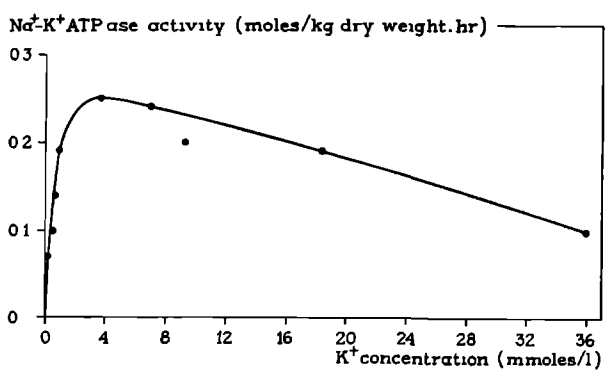


Fig 3 Effect of K^+ concentration on Na^+-K^+ ATPase activity in dog pancreas homogenates. Enzyme activity was measured in medium B, to which KCl was added in final concentrations of 0 to 36 mM. The Na^+ concentration was kept constant (63 mM). The Na^+-K^+ ATPase activity was calculated from the difference between activities in medium B (no K^+) and the media containing KCl

The K^+-activation curve is given in Fig. 3. The concentration of sodium was maintained at 63 mM. The Na^+-K^+ ATPase activity was maximal at 3 9 mM K^+. At higher K^+-levels the activity decreased to approximately 60% at 36 mM K^+. The half-maximal activation was reached at 0 6 mM K^+.

Fig 4 shows the Na^--activation curve for the (Na^+-K^+)-activated ATPase system. The concentration of potassium was kept constant at 5 mM. Maximal activity was reached at about 25 to 40 mM Na^+, while at higher Na^+-levels the activity decreased to about 55% at 100 mM Na^+. Half-maximal activation for Na^+ occurred at 13.6 mM Na^+.

The ouabain inhibition curve for Na^+-K^+ ATPase of dog pancreas in the presence of 5 mM K^+ is given in Fig 5 Complete inhibition of the enzyme system occurred at 10 ^{3} M ouabain. The negative logarithm of the half-maximal inhibition concentration was 6 8

The pH-activity curves for (Na^--K^+)-activated ATPase and Mg^{++}-stimulated ATPase are shown in Fig 6. The pH optimum for Mg^{++} ATPase is pH 9 2 and that for Na^+-K^+ ATPase pH 7 1

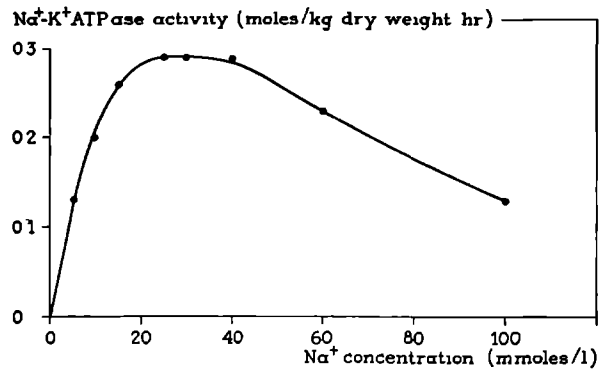


Fig 4 Effect of Na^+ concentration on Na^+-K^+ ATPase activity in dog pancreas homogenates Enzyme activity was measured in medium C to which NaCl was added to give final concentrations of 0 to 100 mM The K^- concentration was kept constant (5mM) The Na^--K^+ ATPase activity was calculated from the difference between activities in medium C (no Na^+) and the media containing NaCl

3 DISCUSSION AND CONCLUSIONS

The data presented in Table 3 and Figs. 2 to 6 demonstrate the presence of an (Na^+-K^+)-activated ATPase system in the dog pancreas, which is very similar to the one previously described by Skou (1957 and 1960) in crab nerve, by Post et al (1960) and Dunham and Glynn (1961) in erythrocyte membranes, by Hokin (1963) and Bonting et al. (1964b) in marine bird nasal gland, by Bonting (1966) in the rectal gland of elasmobranchs and by Bakkeren and Bonting (1968a) in rat

liver. In addition to Mg^{++}, both Na^+ (K_m = 14 mM) and K^+ (K_m = 0.6 mM) are required for activation, while the system is inhibited by the cardiac glycoside ouabain (pI_{50} = 6.8). The pH optimum for the (Na^+-K^+)-activated ATPase system is found at pH 7.1, similar to the pH optima of 7.0 for spiny dog fish rectal gland (Bonting, 1966), of 7.2 in herring gull nasal gland (Bonting et al., 1964b), of 7.3 in lens epithelium (Bonting et al., 1963) and of 7.3 in rat liver (Bakkeren and Bonting, 1968a). Mg^{++}-stimulated ATPase had a pH optimum at pH 9.2, different from that of Na^+-K^+ ATPase, and close to the pH optima of 8.7 in herring gull salt gland, of 8.7 in rat liver and of 8.8 to 8.9 in rectal gland of elasmobranchs, but slightly higher than the pH optimum of 8.4 in rabbit lens epithelium.

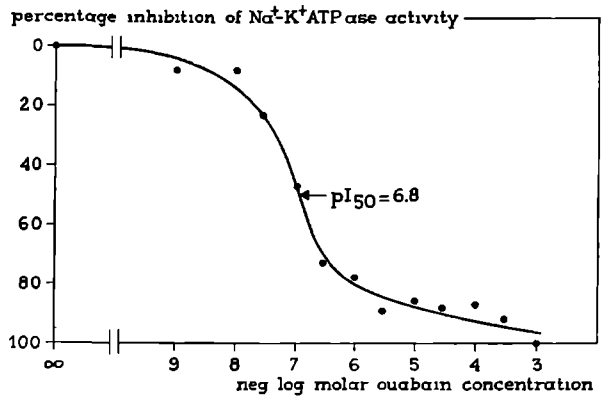


Fig 5 Inhibition of Na^+-K^+ ATPase activity in dog pancreas homogenates by ouabain Enzyme activity was measured in medium A, to which ouabain was added in final concentrations of 10^{-10} to 10^{-3} M and in medium E The Na^+-K^+ ATPase activity was calculated from the difference between activities in medium E and the media containing ouabain In order to calculate the percentage inhibition of the Na^+-K^+ ATPase activity for each inhibitor concentration, the difference between activities in media A (no ouabain) and E was set at 100 The pI_{50} value is the negative logarithm of the ouabain concentration giving 50% inhibition

The absolute Na^+-K^+ ATPase activity in dog pancreas of 0.33 moles ATP hydrolyzed per kg dry weight per hour is similar to the activity found in our laboratory for cat pancreas of 0.36 moles ATP hydrolyzed per kg dry weight per hour. On the one hand, the Na^+-K^+ ATPase activity for dog and cat pancreas is rather low, when compared with the activities for rabbit kidney of 1.12 moles ATP hydrolyzed per kg wet weight per hour, for rabbit brain of 1.33 moles ATP hydrolyzed per kg wet weight per hour (Bonting et al., 1964a) and for cat choroid plexus of 1.9 moles ATP hydrolyzed per kg dry weight per hour (Vates et al., 1964). On the other hand, the activity for dog and cat pancreas is comparable with the activities of 0.22 moles ATP hydrolyzed per kg

wet weight per hour for ciliary body (Bonting and Becker, 1964) and of 0.37 moles ATP hydrolyzed per kg dry weight per hour for rat liver (Bakkeren and Bonting, 1968a). Somogyi (1967) demonstrated that a 10-min. and a 30-min. incubation of an (Na^+-K^+)-activated ATPase preparation with 20 µg trypsin and 300 µg chymotrypsin respectively, in a Tris chloride buffer (pH 7.4 and 8.0 respectively) at 25° C, decreased the ATPase activity by approximately 50%. Thus, we had to consider the possibility that pancreatic (Na^+-K^+)-activated ATPase is inhibited by these proteases present in homogenates of the organ. Since these proteases are only present in an inactive form in pancreas homogenates (trypsinogen and chymotrypsinogen), it is unlikely that they could cause appreciable inactivation of pancreatic Na^+-K^+ ATPase during the one hour incubation at 37° C in the various substrate media.

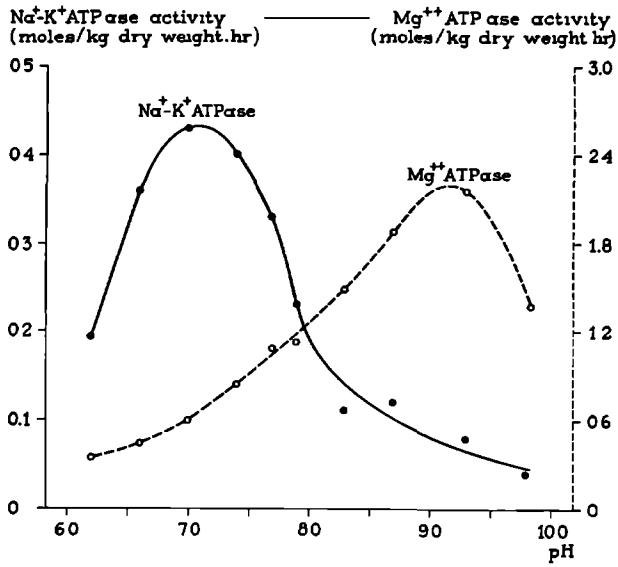


Fig 6 Effect of pH on the activities of Mg^{++} ATPase (o - - - - o) and Na^+-K^+ ATPase (● ——— ●) in dog pancreas homogenates Enzyme activities were measured in media A and E at pH values varying from 6 2 to 9 8 For the pH range from 6 2 to 7 4 the media were prepared with Tris-histidine buffer (50 mM each), while for the pH range from 7 4 to 9 8 Tris-HCl buffer (100 mM) was used The pH of each resulting medium was measured and used in plotting the assay results The Mg^{--} ATPase activity was calculated from the activity in medium E and the Na^+-K^+ ATPase activity from the difference between the activities in media A and E

Once the presence of the (Na^+-K^-)-activated ATPase system in the dog pancreas had been established, the second subject of study has been to investigate the effect of ouabain on the in vivo secretion of fluid and electrolytes in the dog. A detailed report of this study is given in the next chapter.

(Na^+-K^+)-ACTIVATED ADENOSINE TRIPHOSPHATASE AND EXOCRINE PANCREATIC SECRETION IN VIVO IN THE DOG

INTRODUCTION

The ductular cells of the pancreas produce a fluid with sodium and potassium concentrations approximately equal to those in plasma. These cation concentrations are independent of the rate of flow (Johnston and Ball, 1930; Thomas, 1950; Solomon, 1952; Dreiling and Janowitz, 1956). As previously mentioned, the concentrations of calcium and magnesium in pancreatic juice are much lower than in plasma (chapter I, section 2). Bicarbonate and chloride concentrations on the other hand vary in a reciprocal way with the flow rate: with increasing flow rate the concentration of bicarbonate goes up and that of chloride goes down, while the sum of both concentrations remains relatively constant (Hart and Thomas, 1945; Dreiling and Janowitz, 1959; Christodoupoulos et al., 1961).

A role for carbonic anhydrase in the secretion mechanism of pancreatic fluid was suggested by the high, flow-dependent concentration of bicarbonate and the presence of carbonic anhydrase in rather high activity in pancreatic tissue (van Goor, 1948). The decrease in both bicarbonate concentration and flow rate after intravenous administration of acetazolamide, a specific carbonic anhydrase inhibitor, appeared to support such a role (Birnbaum and Hollander, 1953; Dreiling and Janowitz, 1959; Pratt and Alkawa, 1962; Rawls et al., 1963; Pak et al., 1966). An active transport of bicarbonate was postulated, assuming a passive movement of the other ions and of water. However, Rawls et al. (1963) showed that an inhibition of more than 99% of pancreatic carbonic anhydrase activity was required to give a maximal flow inhibition of 50%. This strongly suggests that the action of the enzyme system cannot be the primary, rate-limiting process in the secretion of pancreatic fluid.

In recent years it has become more and more evident that active sodium transport by means of the (Na^+-K^-)-activated ATPase system plays a primary role in the secretion of water and electrolytes by organs with an exocrine function. This is the case in the secretion of aqueous humour (Simon et al., 1962; Bonting and Becker, 1964), cerebrospinal

fluid (Vates et al., 1964), saliva (Schwartz and Matsui, 1967) and sweat (Gibbs et al., 1967; Slegers, 1968). In these cases water appears to follow the actively extruded sodium passively, probably through local osmosis, as previously shown by Diamond (1962) for the isotonic water reabsorption in the rabbit gall bladder.

The constancy of the sodium concentration in pancreatic juice over widely differing flow rates, as found by several investigators, suggested that in exocrine pancreatic secretion a similar mechanism might be the primary and rate-limiting step. In an attempt to elucidate the possible role of the ouabain-sensitive (Na^+-K^+)-activated ATPase system in exocrine pancreatic fluid and electrolyte secretion, we could demonstrate the presence of the enzyme system in homogenates of dog pancreas (chapter IV). In order to establish the relationship between the enzyme system and the secretory system in the pancreas, we investigated the effect of the cardiac glycoside ouabain on pancreatic fluid and electrolyte secretion in vivo in the dog. The experimental results are described and discussed in this chapter.

1. PREPARATION OF ANIMALS

In order to investigate the effect of the cardiac glycoside ouabain on exocrine pancreatic fluid secretion in vivo in the dog, fluid and electrolyte secretion was stimulated by perfusing 0.5 units secretin per min. into the left femoral vein and the pancreatic fluid was collected by means of a cannula placed in the main pancreatic duct. Vanamee and collaborators (1966) have shown that a continuous intravenous perfusion of secretin at this rate gives, after an equilibration period, a relatively constant flow rate. Intravenous administration of ouabain in the right femoral vein failed to affect exocrine pancreatic secretion and for that reason we decided to inject the cardiac glycoside directly into the arterial blood supply of the pancreas. For this purpose we shunted the left femoral artery to the celiac axis, the most important arterial source of the pancreas.

Throughout our study we have used male and female mongrel dogs, weighing 8 to 15 kg. The animals were starved for 24 hours before each experiment and they were initially anaesthetized with 15 to 20 mg sodium thiopentone per kg body weight, injected intravenously. During the short period of anaesthesia, following intravenous injection of sodium thiopentone, a tube was placed in the trachea and connected to a semi-closed system provided with a carbon dioxide absorber and a halothane vapourizer. By means of this system controlled amounts of N_2O (2 liter/min.), oxygen (1 liter/min.) and halothane (2-3%) were introduced into the trachea to keep the animals in early stage three of anaesthesia. The left femoral vein was then cannulated by means of polyethylene tubing (1.57 mm inner diameter).

The animal was treated with heparin (2 mg/kg body weight intra-

venously), to prevent blood from clotting in the arterial shunt, which was subsequently placed between the left femoral artery and the celiac axis. Furthermore, all excised blood vessels in operation wounds were coagulated and the surgical wounds were all treated with an active thrombin preparation, Topostasin, to avoid severe bleeding during the experiment. The abdomen was opened through a midline incision and the celiac axis was localized and prepared for the shunt to the exposed left femoral artery. The shunt consisted of a siliconized stainless steel unit, provided with two entrances and a 30-cm long polyethylene catheter (2 0 mm inner diameter). By means of a ligature the stainless steel unit was inserted in the left femoral artery, after which the catheter, provided with a collar, was placed in the celiac axis. In order to avoid the release of endogenous secretin and pancreozymin, the intestine was ligated distally to the pylorus to prevent stomach contents from spilling into the duodenum The major pancreatic duct, which lies several centimeters caudal to the bile duct papilla, was cannulated by means of a 20-cm polyethylene catheter (0 75 mm inner diameter). A small submucosal ligature was placed around the accessory pancreatic duct The operation was terminated by closing the midline incision. Heparinisation was maintained for the duration of the experiment by hourly doses of 1 mg heparin per kg body weight after the first dose The animals were then allowed to rest for 60 to 90 minutes before starting the experiment.

2 RATE OF SECRETION IN THE DOG

In general the animals were rested for 60 to 90 minutes after surgery was ended, before the acute experiment started Each experiment was initiated by connecting the polyethylene catheter, placed in the left femoral vein, to a perfusor (Braun, type 71062). By means of this perfusor a continuous intravenous perfusion of secretin was effected. The perfusate was freshly prepared before each experiment and contained 200 Crick-Harper-Raper units secretin dissolved in 40 ml saline. A total of 0.1 ml solution containing 0.5 units was perfused into the femoral vein per minute. Pancreatic juice was collected through the 20-cm polyethylene catheter, inserted in the main pancreatic duct, in test tubes (10 mm inner diameter and 8 cm height) in 10-min periods. The volume of the fluid was determined by weighing to the nearest 0 1 mg, assuming a specific gravity of 1 01. (Maren, 1956).

Basal pancreatic secretion, i.e. without external secretin stimulation, was determined by collecting pancreatic fluid in the period that the animals were allowed to rest after the operation was terminated It varied between 100 and 550 µl per 10-min. period, indicating that under these conditions the pancreas is still capable of producing pancreatic juice.

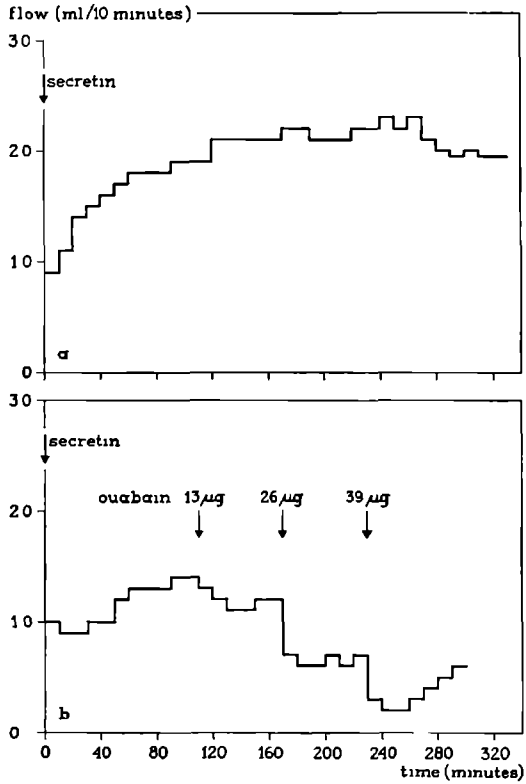


Fig. 7. a Pancreatic fluid secretion in the dog in vivo Flow was stimulated by means of a continuous intravenous secretin perfusion (0 5 units/min) and fluid was collected in 10-min periods. Average results are given for five animals b Effect of cumulative doses of ouabain on stimulated pancreatic fluid secretion in a typical experiment Ouabain was injected into the shunt between the left femoral artery and the celiac axis.

About 5 min. after initiating the continuous intravenous secretin perfusion the flow rate began to increase. Therefore, we waited at least five minutes in all experiments before beginning the collection of pancreatic fluid in 10-min intervals. In all five control experiments the flow increased during the first 90 minutes and then became relatively constant (Fig. 7a). Between 90 and 320 minutes there was an average flow of 2.1 (SE 0.02) ml per 10-min. period.

3 ELECTROLYTE COMPOSITION OF PANCREATIC FLUID

Sodium and potassium determinations were carried out in diluted samples of each 10-min. fraction. Twenty µl of pancreatic juice were diluted with twice distilled water to a final volume of four ml. This

volume was sufficient for flame photometric measurements of both sodium and potassium. A detailed description of the procedure used in this determination is given in chapter III.

Fig. 8a shows the sodium and potassium concentrations of the pancreatic fluid secreted by the control animals. The sodium concentration remained relatively constant, ranging from 155 to 169 mmoles per liter. So did the potassium concentration, which ranged between 4.5 and 5.8 mmoles per liter.

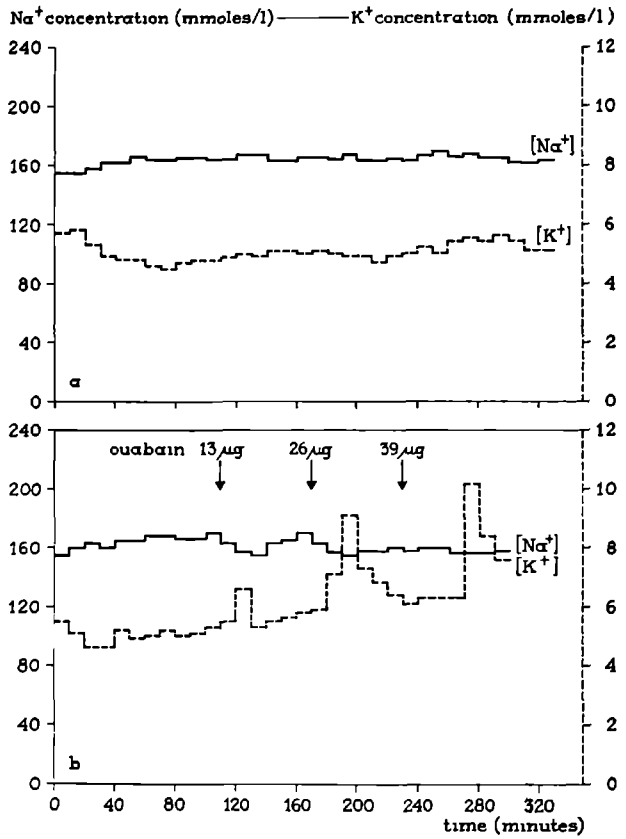


Fig 8 a Concentrations of Na^+ (–) and K^+ (- - - -) in pancreatic fluid of control experiments Average results are given for five animals b Effect of cumulative doses of ouabain on the concentrations of Na^+ (——) and K^+ (- - - -) in pancreatic fluid from a typical experiment Ouabain was injected into the shunt between the left femoral artery and the celiac axis.

Fifteen µl samples were taken from the first four 10-min. period collections and for the duration of the experiment from every second 10-min. fraction for total CO_2 determinations. These samples were

diluted with 150 µl twice distilled water, and 30 µl of the diluted material were used for CO_2 measurements in a Natelson microgasometer. Since the pH of the pancreatic juice ranges between 7.5 and 8.0, the values found for total CO_2 represent approximately the bicarbonate concentrations (Pak et al., 1966). Chloride was determined by the coulometric titration method, as previously described by Cotlove and Nishi (1961). Full details of the procedures followed in the CO_2 and chloride determinations are given in the third chapter.

Fig. 9a gives the average results for bicarbonate and chloride concentrations in control experiments. Bicarbonate concentration increased during the first 40 minutes, reaching a maximum value of approximately 130 mM. There was a slight decrease in bicarbonate concentration towards the end of the experiment. Chloride concentration, however, decreased during the first 40 minutes, reaching a minimum value of about 43 mM, and during the rest of the experiment it tended to increase slightly. This inverse relationship between bicarbonate and chloride concentrations, as found in the control animals, has previously been described by many other authors.

4. EFFECT OF OUABAIN

In acute experiments with dogs we studied the effect of the cardiac glycoside ouabain on exocrine pancreatic fluid and electrolyte secretion. The anaesthetized animals were always allowed to rest for 60 to 90 minutes after surgery and then a continuous intravenous infusion of secretin was instituted. As previously described in this chapter, the flow under control conditions increased for about 90 minutes and then became relatively constant. Therefore, we waited at least 90 minutes before injecting ouabain into the animals.

In a first series of experiments we used six animals to study the effect of ouabain, injected intravenously, on exocrine pancreatic secretion.

Intravenous injection of 50 to 70 µg ouabain per kg body weight in three animals gave no significant flow inhibition. Intravenous administration of larger doses of the cardiac glycoside in three other animals, namely 100 to 120 µg ouabain per kg body weight, caused arrhythmia within a few minutes, leading to circulatory deficiency and finally to the death of two animals within 45 minutes. Flow inhibition in these animals could, therefore, not be attributed to direct inhibition of pancreatic secretion alone, since severe circulatory effects occurred simultaneously.

In another series of three experiments an attempt was made to inject ouabain directly into the arterial blood supply of the organ. For this purpose a radio-opaque catheter was passed under fluoroscopy through the left femoral artery into the aorta and inserted at the level of the diaphragm, with its tip bent in the direction of the celiac axis. By means of this catheter it was possible to inject ouabain directly into the arterial

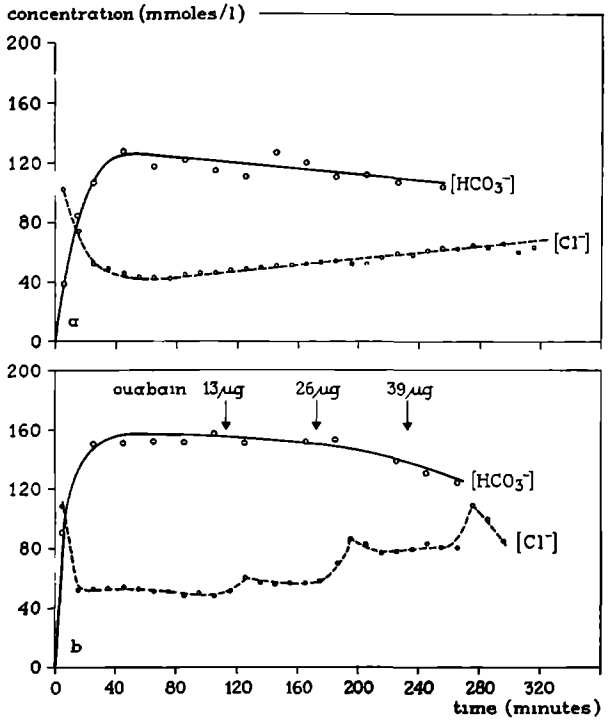


Fig 9 a Concentrations of HCO_3^- (o – – o) and Cl^- (o - - - - o) in pancreatic fluid of control experiments Average results are given for five animals b Effect of cumulative doses of ouabain on the concentrations of HCO_3^- (o — o) and Cl^- (o - - - - o) in pancreatic fluid from a typical experiment Ouabain was injected into the shunt between the left femoral artery and the celiac axis

blood supply of the pancreas. Administration of 50 to 70 µg ouabain per kg body weight in this way resulted in an average flow inhibition of 45% in three experiments.

These results were encouraging, though not fully satisfactory. Nevertheless, they suggested to us the idea of shunting the left femoral artery to the celiac axis, which is the most important circulatory source of the pancreas (Chapter I, section 1). Varying doses of ouabain could now be injected directly into the shunt. A series of eleven experiments was carried out in this way. Fig. 7b shows the results obtained in a typical experiment. In the latter experiment doses of 13 µg per kg body weight were injected into the shunt between the left femoral artery and the celiac axis, resulting in 15, 55 and 86% flow inhibition after cumulative doses of 13, 26 and 39 µg per kg body weight respectively. After ouabain administration the sodium concentration of the pancreatic fluid was not significantly altered, while the potassium concentration showed a transient increase which persisted in part (Fig. 8b). In Fig. 9b the

effects on bicarbonate and chloride concentrations are presented. After ouabain injection the bicarbonate concentration remained virtually unchanged, while the chloride concentration showed a transient increase, which was larger than the corresponding increase in the potassium level. The sum of the sodium and potassium concentrations before ouabain administration was 169.3 mmoles per liter and after injection of the cardiac glycoside it was 165.5 mmoles per liter. The sum of the concentrations of bicarbonate and chloride also remained relatively unchanged (205.8 and 204.1 mmoles/liter respectively) after ouabain.

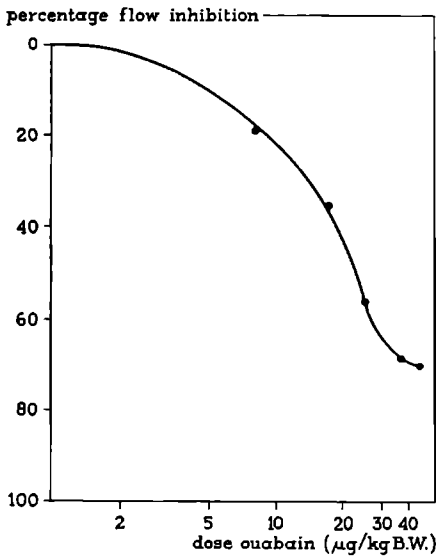


Fig.10. Inhibition of stimulated fluid secretion in the dog in vivo by ouabain. Varying doses of ouabain (7 to 45 μg/kg body weight) were injected into the shunt between the left femoral artery and the celiac axis. Average results are given for eleven animals.

In order to obtain a flow inhibition curve in vivo, varying doses of ouabain, ranging from 7 to 45 μg per kg body weight, were injected into the shunt in a total of eleven animals, and the average results for five different doses of ouabain are given in Fig. 10. A maximal average flow inhibition of 70% was seen after 45 μg per kg body weight.

The S-shaped inhibition curve resembles that for the enzyme system. Unfortunately, we could not express the dose of ouabain injected into the animals in moles per liter on the active site and for this reason we cannot compare the pI_{50} value found for the $(Na^+\text{-}K^+)$-activated ATPase system to the dose of injected ouabain causing a 50% flow inhibition.

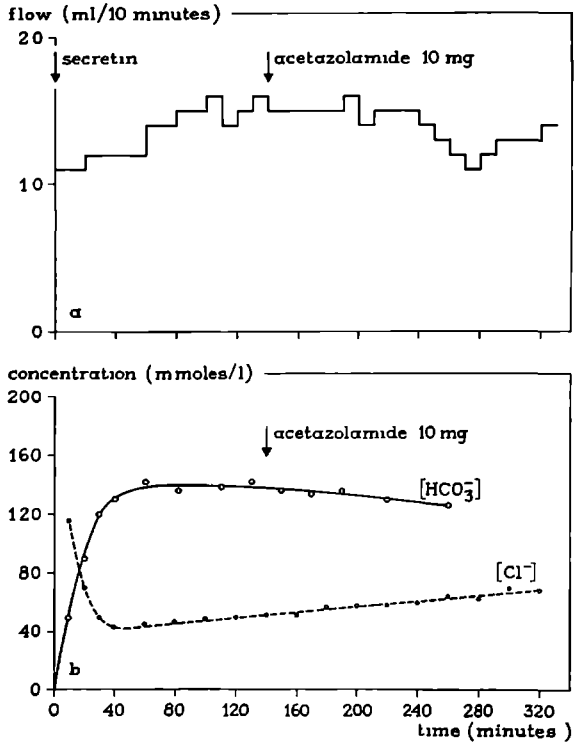


Fig 11 a Effect of acetazolamide on stimulated pancreatic fluid secretion in a typical experiment Acetazolamide was injected into the shunt between the left femoral artery and the celiac axis b Effect of acetazolamide on the concentrations of HCO_3^- (o —— o) and Cl^- (o - - - - o) in pancreatic fluid from a typical experiment

5 EFFECT OF ACETAZOLAMIDE

An investigation was made of the effect of acetazolamide, a specific carbonic anhydrase inhibitor, on pancreatic fluid and electrolyte secretion, stimulated by a continuous intravenous perfusion of secretin. The drug was dissolved in saline and injected directly into the shunt which was prepared between the left femoral artery and the celiac axis in two animals Injection of 10 mg acetazolamide per kg body weight resulted in a maximal flow inhibition of 23 and 27%, average 25%, about 150 minutes after administration of the drug. Sodium and potassium concentrations were not affected after acetazolamide injection. Bicarbonate concentration showed a slight decrease, while chloride concentration increased a little. Fig. 11 represents the flow rate and the concentrations of bicarbonate and chloride in the second of these two experiments.

6 DISCUSSION AND CONCLUSIONS

The presence of the Na^+-K^+ ATPase system in homogenates of dog

pancreas and the similarity of its properties to those found for the enzyme system in several other tissues (Chapter IV), suggest that this enzyme system might play a part in the exocrine secretion of the pancreas. This surmise is supported by the inhibitory effect of ouabain on pancreatic flow. In the shunt experiments, ouabain administration in varying doses ranging from 7 to 45 μg ouabain per kg body weight, resulted in 19 to 70% flow inhibition, with a maximal average inhibition of 70% after 45 μg per kg body weight. A similar inhibition of flow caused by ouabain had previously been found for aqueous humour and cerebrospinal fluid. The maximal average flow inhibition of 70% was also the maximal flow inhibition found for aqueous humour, when 0.5 mg ouabain was injected into the vitreous humour of one eye of the rabbit In the latter case this could be attributed to the finding that about 70% of aqueous humour flow is due to secretion and the rest to ultrafiltration (Cole, 1960a and 1960b; Kinsey, 1960).

After stimulation of flow by a continuous intravenous secretin perfusion, the concentrations of sodium and potassium remained unchanged. Bicarbonate concentration increased and reached values 5 to 6 times higher than in plasma, in agreement with the results obtained by several groups of investigators. The concentration of chloride, however, decreased to a minimum of 43 mM and showed a tendency to increase towards the end of the experiment.

After ouabain administration no change was seen, either in the sodium concentration or in the bicarbonate concentration. Most likely the increase of the potassium concentration is due to a leakage of intracellular K^+ into the duct, after inhibition by ouabain of the sodium pump on the lumen side of the exocrine cells of the pancreas. Increase in potassium concentration was also observed in aqueous humour after inhibition of flow by ouabain (Bonting and Becker, 1964) and in rat bile after ouabain was injected into the portal vein (Bakkeren, 1968). The 30% of pancreatic flow, which could not be inhibited by ouabain, may represent ultrafiltration as in the case of aqueous humour, but there is no morphological evidence for ultrafiltration in the pancreas. It might also be due to the fact that part of the organ is not reached by injection of ouabain via the shunt between the left femoral artery and the celiac axis, as found by Colwell and Colwell (1959). They showed that an experimental solution (methylene blue dye) introduced into the celiac axis is carried into the body and some portions only of the head and the tail of the pancreas.

It has been shown that active sodium transport by means of the (Na^+-K^+)-activated ATPase system is the primary and rate-limiting process in the secretion of aqueous humour in the rabbit, in the formation of cerebrospinal fluid in the cat and in the secretion of sweat in the cat, with the sodium pump being identical with or very closely related to the (Na^+-K^+)-activated ATPase system. Ouabain, a cardiac glyco-

side characterized by the unsaturated lactone group, apparently exerts its inhibitory influence primarily on the secretion of sodium, as demonstrated for squid axon (Caldwell and Keynes, 1959; Baker, 1964), the erythrocyte (Post et al., 1960; Dunham and Glynn, 1961; Hoffman, 1962a), the salt gland of marine birds (Thesleff and Schmidt-Nielsen, 1962; Hokin, 1963; Bonting et al., 1964b) and the choroid plexus (Vates et al., 1964). In these cases, water follows the actively secreted sodium passively, possibly through local osmosis, as shown by Diamond (1962) for the isotonic water reabsorption in the gall bladder. In all these cases ouabain influences the total output only. It does not affect the concentration of sodium in the transported fluid. The flow inhibition by ouabain observed in our experiments and the constancy of the Na^+ concentration are consistent with the assumption that in pancreatic secretion also active sodium extrusion is the primary and rate-limiting step.

From the average control flow (12 ml/hr), the average Na^+ concentration of the secreted fluid (162 meq/l) and the average Na^+-K^+ ATPase activity (0.33 moles ATP hydrolyzed per kg dry wt per hr; total wet wt of pancreas 10 g; dry wt/wet wt ratio 0.30) the molar ratio of Na^+ secreted to ATP hydrolyzed was calculated to be 1.8. This ratio falls within the range of values (1.8-3.1) quoted by Bonting (1966) for a variety of tissues. This reinforces our tentative conclusion that fluid and electrolyte secretion by the pancreas are mediated by the Na^+-K^+ ATPase system. It is not surprising that the value for the pancreas is rather low, since part of the Na^+-K^+ ATPase activity is likely to be present in cells not involved in fluid and electrolyte secretion.

Injection of 10 mg acetazolamide per kg body weight into the arterial blood supply of the pancreas resulted in a 25% inhibition of flow rate. This contrasts with the earlier findings of many other investigators, who found a maximal flow inhibition of approximately 50% after intravenous administration of acetazolamide. This discrepancy can at least partly be accounted for by the differences in methods used in studying the effect of acetazolamide on stimulated pancreatic fluid and electrolyte secretion.

While the results presented in this chapter suggest that the (Na^+-K^+)-stimulated ATPase system plays a part in exocrine pancreatic secretion, the technical limitations of the in vivo approach made it desirable to carry out in vitro studies on the isolated organ in order to arrive at more definite conclusions. This approach and the results obtained with it are discussed in the next two chapters. Chapter VI deals with the occurrence and properties of the (Na^+-K^+)-activated ATPase system in rabbit pancreas, while chapter VII describes the experiments on fluid and electrolyte secretion.

OCCURRENCE OF AN (Na^+-K^+)-ACTIVATED ATPase SYSTEM IN RABBIT PANCREAS

INTRODUCTION

As in the case of the dog it was necessary to demonstrate first of all the presence of a ouabain-sensitive (Na^+-K^+)-activated ATPase system in homogenates of rabbit pancreas. Once the enzyme system had been demonstrated its characteristics were determined in order to compare them with those of the secretory system.

1. ACTIVITIES OF ATPase

Table 4 lists the relative ATPase activities in the various substrate media and the absolute activities for both Mg^{++}-stimulated ATPase and (Na^+-K^+)-activated ATPase for rabbit pancreas homogenate. Omission of potassium and sodium or addition of 10^{-4} M ouabain resulted in an average inhibition of 8.3% of ATPase activity, indicating that the inhibited ATPase activity represents the ouabain-sensitive (Na^+-K^+)-activated ATPase. The inhibition in medium C (sodium-free) was somewhat higher than in the other media. This is probably due to a slight sodium-sensitivity of the Mg^{++}-stimulated ATPase, as previously described for a large number of tissues of several other species. The inhibition in medium B (potassium-free) is incomplete, presumably due to a slight activation of the (Na^+-K^+)-activated ATPase by small amounts of tissue K^+ present in the incubation mixture (about 0.2 mM K^+), since the half-maximal activation concentration of K^+ is only 0.8 mM (Fig. 13). The best values for Na^+-K^+ ATPase activity should therefore be obtained from the difference between the activities in medium A and the media D and E.

The average Na^+-K^+ ATPase activity in rabbit pancreas was 0.23 (SE: 0.029) and for Mg^{++} ATPase an average of 2.74 (SE: 0.206) was found, both expressed in moles ATP hydrolyzed per kg dry weight of tissue per hour at 37° C.

Since the relative (Na^+-K^+)-stimulated ATPase activity was rather low and this activity must be determined by a differential assay, difficulties were encountered in studying the properties of the enzyme system e.g. sodium and potassium activation, ouabain inhibition and pH dependence. Therefore a pretreatment of the enzyme preparation with

Table 4

RELATIVE AND ABSOLUTE ATPase ACTIVITIES FOR RABBIT PANCREAS

Medium	Relative ATPase activity %	
	No pretreatment	After urea pretreatment
A (complete)	100	100
B (no K^+)	95 5 ± 0 7	87 5 ± 0 7
C (no Na^+)	83 9 ± 2 9	71 3 ± 3 2
D (10^{-4} M ouabain)	93 5 ± 1 0	87 1 ± 1 3
E (no K^+, 10^{-4} M ouabain)	93 7 ± 1 7	90 6 ± 3 4
Average of media B, C, D, E	91 7 ± 2 6	84 1 ± 4 3
Absolute activities in moles ATP hydrolyzed /kg dry wt /hr		
Mg^{++} ATPase	2 74 ± 0 206	1 22 ± 0 160
Na^+-K^+ ATPase	0 23 ± 0 029	0 22 ± 0 014

ATPase activity in medium A (complete medium) set at 100, data for media B, C, D and E (means with standard errors for three determinations) indicate the activity remaining after inhibition of the Na^+-K^+ ATPase activity in various ways The absolute activities for Mg^{++}-stimulated and (Na^+-K^+)-activated ATPase are given as means with standard errors for 6 determinations for both urea-treated and untreated preparations

a concentrated urea solution was carried out By this method, as described by Glynn et al (1965) and by Skou and Hilberg (1965) the Mg^{++} ATPase activity decreases considerably, while the (Na^+-K^+)-stimulated ATPase activity does not change. In the case of rabbit pancreas there was a decrease in the Mg^{++}-stimulated ATPase activity of approximately 56%, while the Na^+-K^+ ATPase activity remained unchanged This increased the percentage Na^+-K^+ ATPase activity related to total ATPase from 8 to 16% (Table 4)

2 PROPERTIES OF THE ENZYME SYSTEM

The effect of increasing the Mg^{++} concentration from 0 to 6 mM, while the ATP-level was maintained at 2 mM is shown in Fig 12. In the absence of Mg^{++} there was virtually no ATPase activity Mg^{++}-stimulated ATPase activity was maximal at 2 mM Mg^{++}, approximately 18% higher than at the routinely used level of 1 mM Mg^{++}.

Na^+-K^+ ATPase activity was maximal at 2 to 4 mM Mg^{++} and began to decrease at 6 mM Mg^{++}. Thus the optimal Mg^{++}/ATP ratio for the Na^+-K^+ ATPase system was 1

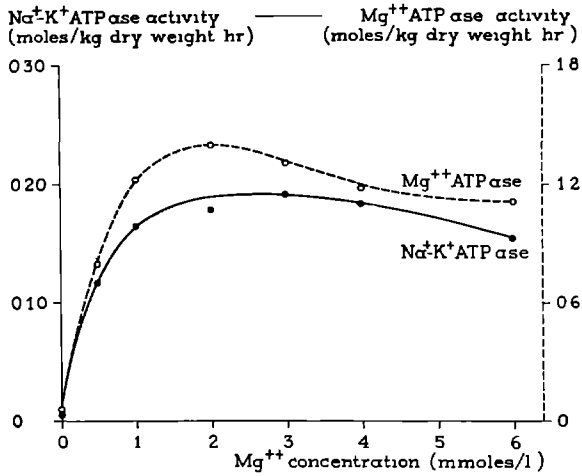


Fig 12 Effect of Mg^{++} concentration on the activities of Mg^{++} ATPase (o - - - - o) and Na^+-K^+ ATPase (● – – ●) in rabbit pancreas homogenates after urea pretreatment Enzyme activities were measured in media A and E (each containing 2 mM ATP) in which the Mg^{++} concentration was varied from 0 to 6 mM The Mg^{++} ATPase activity was calculated from the activity in medium E, the Na^+-K^+ ATPase activity from the difference between the activities in media A and E

The K^+-activation curve is given in Fig. 13. The Na^+ concentration was kept constant at 63 mM. The Na^+-K^+ ATPase activity was maximal at 4 mM K^-. At higher potassium levels the activity decreased to approximately 55% at 18 mM K^+ Half-maximal activation was reached at 0.8 mM K^+.

Fig. 14 shows the Na^+-activation curve for the (Na^--K^+)-activated ATPase system. The K^+ concentration was kept constant at 5 mM Maximal activity was reached at 25 mM Na^+, while at higher sodium concentrations the Na^+-K^+ ATPase activity decreased to about 60% at 80 mM Na^+. Half-maximal activation for Na^+ occurred at 10 mM Na^+

The decrease in Na^+-K^+ ATPase activity in the K^+- and Na^+-activation curves at higher concentrations of the activating cation, observed both in dog pancreas (Figs. 3 and 4) and rabbit pancreas (Figs 13 and 14) have in other tissues never been observed in such a pronounced form. They might be due to a decrease in the Mg^{++} ATPase activity, for which the method used offered no correction. In view of the high proportion of Mg^{++} ATPase activity, such an effect might lead to a

considerable effect on the calculated Na^+-K^+ ATPase activity. Since we were interested mainly in the rising slope of the curves and the maximal activation concentrations, no attempt was made to clarify this point.

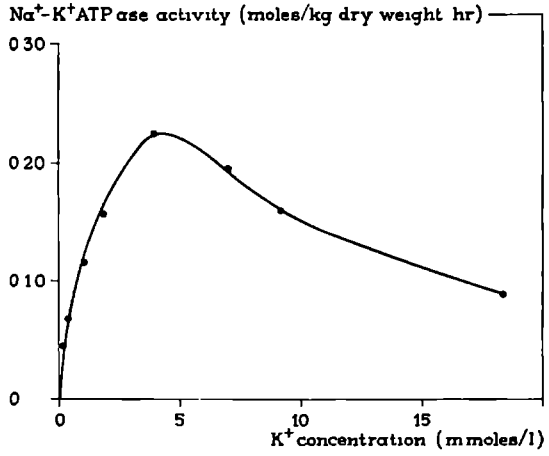


Fig 13 Effect of K^+ concentration on Na^+-K^+ ATPase activity in rabbit pancreas homogenates after urea pretreatment Enzyme activity was measured in medium B, to which KCl was added in final concentration of 0 to 18 mM The Na^+ concentration was kept constant (63 mM) The Na^+-K^+ ATPase activity was calculated from the difference between activities in medium B (no K^+) and the media containing KCl.

The ouabain and erythrophleine inhibition curves for (Na^+-K^+)-activated ATPase of rabbit pancreas in the presence of 5 mM K^+ are shown in Fig. 15. (Na^+-K^+)-stimulated ATPase activity was totally inhibited at 3.10^{-3} M ouabain and 10^{-4} M erythrophleine respectively. The negative logarithms of the half-maximal inhibition concentrations (pI_{50}) for ouabain and erythrophleine were 5.4 and 5.8 respectively. At 10^{-9} M ouabain a stimulatory effect of 16% (SE: 2.0) was observed, while 10^{-10} M ouabain failed to affect the (Na^+-K^+)-stimulated ATPase activity.

The effect of varying the pH from 6.2 to 10.0 is given in Fig. 16. From these pH-activity curves for both Na^+-K^+ ATPase and Mg^{++} ATPase it is evident that the two enzyme systems have different pH optima. The optimum for the Mg^{++}-stimulated ATPase system is at pH 8.8 and that for the (Na^+-K^+)-activated ATPase system at pH 7.2.

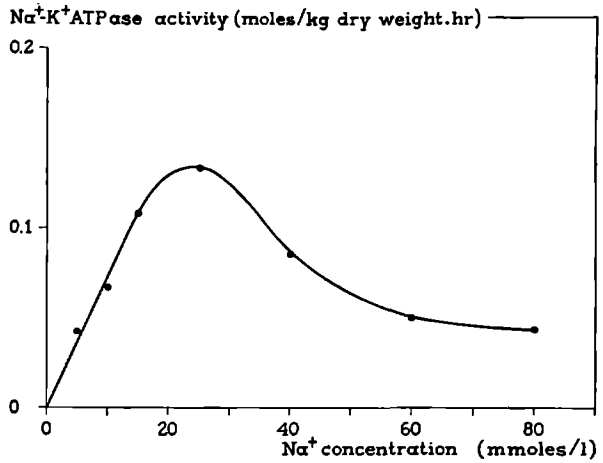


Fig. 14. Effect of Na^+ concentration on Na^+-K^+ ATPase activity in rabbit pancreas homogenates after urea pretreatment. Enzyme activity was measured in medium C, to which NaCl was added in final concentrations from 0 to 80 mM. The K^+ concentration was kept constant (5 mM). The Na^+-K^+ ATPase activity was calculated from the difference between activities in medium C (no Na^+) and the media containing NaCl.

3. EFFECT OF ACTIVE TRANSPORT INHIBITORS

Both cardiac glycosides and Erythrophleum alkaloids are capable of inhibiting the (Na^+-K^+)-activated ATPase system (Bonting et al., 1964a). The inhibition curves for the cardiotonic steroid ouabain and the Erythrophleum alkaloid erythrophleine are shown in Fig. 15. Table 5 shows the relative inhibition of the Na^+-K^+ ATPase activity by several cardiac glycosides and erythrophleine, all in a 10^{-4} M concentration. At this concentration only erythrophleine inhibited the (Na^+-K^+)-stimulated ATPase activity completely, while for ouabain and scillaren A, inhibitions of 74 and 64% respectively were observed. Apparently the enzyme system is less sensitive to digoxin (52%). Hexahydroscillaren A in the same concentration on the other hand failed to inhibit the Na^+-K^+ ATPase activity. The lack of inhibitory activity of this cardiac glycoside derivative is most likely due to its saturated lactone group (Dunham and Glynn, 1961). The inhibitory effects of these inhibitors on fluid and electrolyte secretion by the isolated rabbit pancreas are reported in the next chapter (section 5).

4. DISCUSSION AND CONCLUSIONS

The data presented in this chapter (Tables 4 to 5 and Figs. 12 to 16) demonstrate the presence of an (Na^+-K^+)-activated ATPase system in

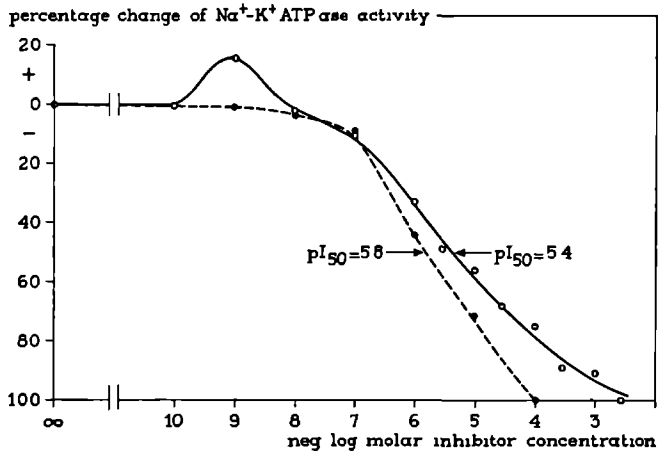


Fig 15 Effects of ouabain (o——o) and erythrophleine (● - - - - ●) on Na^+-K^+ ATPase activity in rabbit pancreas homogenates after urea pretreatment Enzyme activity was measured in medium A to which graded amounts of inhibitor was added in final concentrations of 10^{-9} to 10^{-3} M and in medium E The Na^+-K^+ ATPase activity was calculated from the difference between activities in medium E and the media containing inhibitor Percentage inhibition of Na^+-K^+ ATPase activity for each inhibitor concentration was calculated by setting the difference between activities in media A (no inhibitor) and E at 100 The pI_{50} value is the negative logarithm of the inhibitor concentration giving 50% inhibition

the rabbit pancreas similar to that previously described in crab nerve (Skou, 1957 and 1960), in erythrocyte membranes (Post et al., 1960, Dunham and Glynn, 1961), in marine bird nasal gland (Hokin, 1963, Bonting et al , 1964b), in the rectal gland of elasmobranchs (Bonting, 1966) and in dog pancreas (chapter IV, section 1, Ridderstap and Bonting, 1969a) In addition to Mg^{++}, both Na^+ (K_m = 10 mM) and K^+ (K_m = 0 8 mM) are required for activation, while the enzyme system is inhibited by cardiac glycosides (ouabain, scillaren A and digoxin) and the Erythrophleum alkaloid erythrophleine For ouabain the pI_{50} is 5 4, while in dog pancreas a much higher value was found (pI_{50} = 6 8), indicating that the rabbit pancreas is less sensitive to ouabain than the dog pancreas The pH optimum for the (Na^+-K^+)-activated ATPase system is found at pH 7.2, similar to the pH optima of 7.2 in herring gull nasal gland (Bonting et al., 1964b), of 7 3 in lens epithelium (Bonting et al., 1963), of 7.3 in rat liver (Bakkeren and Bonting, 1968a) and of 7.1 in dog pancreas (chapter IV, section 2, Ridderstap and Bonting, 1969a) The Mg^{++}-stimulated ATPase system has a pH optimum at pH 8.8, different from that of the (Na^+-K^+)-activated ATPase enzyme system, but close to the pH optima of 8.7 in herring gull salt gland (Bonting et al., 1964b), of 8 8-8 9 in rectal

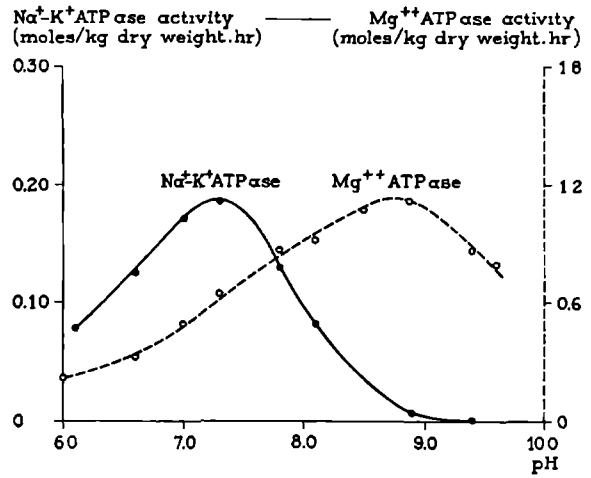


Fig. 16. Effect of pH on the activities of Mg^{++} ATPase (o - - - - o) and Na^{+}-K^{+} ATPase (● —— ●) in rabbit pancreas homogenates after urea pretreatment. Enzyme activities were measured in media A and E at pH values varying from 6.2 to 9.5. For the pH range from 6.2 to 7.4 the media were prepared with Tris-histidine buffer (each 50 mM), while for the pH range from 7.4 to 9.5 Tris-HCl buffer (100 mM) was used. The pH of each resulting medium was measured and used in plotting the assay results. The Mg^{++} ATPase activity was calculated from medium E, the Na^{+}-K^{+} ATPase activity from the difference in activities in media A and E.

gland of elasmobranchs (Bonting, 1966), of 8.7 in rat liver (Bakkeren and Bonting, 1968a), but slightly higher than the pH optimum of 8.4 in rabbit lens epithelium (Bonting et al., 1963) and somewhat lower

Table 5

EFFECTS OF ACTIVE TRANSPORT INHIBITORS ON Na^{+}-K^{+} ATPase ACTIVITY

Inhibitor (10^{-4} M)	Inhibition of Na^{+}-K^{+} ATPase activity, %
Erythrophleine	100
Ouabain	74 ± 2.8
Digoxin	52 ± 1.9
Scillaren A	64 ± 3.2
Hexahydroscillaren A	0

Percent changes are given as means ± SE for determinations in triplicate of three preparations.

than the pH optimum of 9.2 in dog pancreas (chapter IV, section 2; Ridderstap and Bonting, 1969a). The (Na^+-K^+)-stimulated ATPase activity of 0.23 moles ATP hydrolyzed per kg dry weight per hour is close to the activity of 0.33 moles ATP hydrolyzed per kg dry weight per hour in dog pancreas. This activity is rather low compared to those found by Bonting et al. (1964a) in rabbit kidney and brain (1.12 and 1.33 M/kg wet weight/hr respectively), but is comparable to the activity of 0.22 moles ATP hydrolyzed per kg wet weight per hour in rabbit ciliary body (Bonting and Becker, 1964).

THE SODIUM PUMP AND EXOCRINE SECRETION FROM THE ISOLATED RABBIT PANCREAS

INTRODUCTION

In an attempt to elucidate the role of the $(Na^+\text{-}K^+)$-activated ATPase system in exocrine pancreatic secretion we studied first the pancreas of the anaesthetized dog in situ. As already described in chapters IV and VI an adenosine triphosphatase, activated by sodium and potassium and inhibited by ouabain, was demonstrated in homogenates of dog and rabbit pancreas. The cardiac glycoside ouabain also inhibited the in vivo pancreatic fluid secretion in the dog, with a maximal average flow inhibition of approximately 70% occurring after injection of 45 μg inhibitor per kg body weight directly into the arterial blood supply of the organ. The concentrations of sodium and bicarbonate in the secreted fluid were not altered after ouabain administration, while the potassium and chloride concentrations showed small largely transient increases. While these findings suggest that the $(Na^+\text{-}K^+)$-activated ATPase system plays a part in exocrine pancreatic secretion, the technical limitations of the in vivo approach made it desirable to carry out in vitro experiments on the isolated organ in order to obtain further evidence.

The rabbit pancreas, because of its very thin sheet-like configuration, is very suited for in vitro studies. The organ is interposed between the ascending and descending limbs of the first intestinal loop and it can very easily be isolated as a whole and placed in a special chamber to which a balanced salt solution is added. Rothman (1964) showed that the isolated pancreas, incubated in a Krebs-Henseleit solution, secretes a fluid of nearly the same electrolyte composition as in situ and at a flow rate which is relatively constant for at least five hours after isolation and which is about six times higher than in situ. The use of this preparation eliminates circulatory, hormonal and nervous effects on secretion, while the concentrations of chemical agents added to the bathing solution are exactly known and the ionic environment can be varied at will.

First of all we studied the basal secretion (i.e. without external secretin stimulation) of fluid and electrolytes by the isolated rabbit pancreas. Special attention was paid to the effect of ouabain and other specific inhibitors of active transport on fluid and ^{22}Na secretion and

the effect of a low sodium environment on fluid and electrolyte secretion. Also the direct effect of the carbonic anhydrase inhibitor acetazolamide and the combined effect of this drug and ouabain on exocrine pancreatic secretion were investigated; the effects of anaerobiosis, metabolic inhibitors and secretin on water and electrolyte secretion by the isolated rabbit pancreas were studied too. At the end of this chapter the properties of the (Na^+-K^+)-activated ATPase system in rabbit pancreas (chapter VI) are compared with those found for the secretory system.

1 PREPARATION OF THE ORGAN

For studying exocrine pancreatic secretion in vitro we needed a pancreas that would permit collection of the secreted fluid and adequate oxygenation simultaneously. Rothman (1964) proved that the pancreas of the rabbit meets both criteria. Since the organ is entirely located within the mesentery of the first intestinal loop, also called the "mesomental loop" (Craigie, 1948), it can easily be isolated. Adequate oxygenation of the organ is quite possible because the rabbit pancreas is nearly as thin as the surrounding mesentery (about 0.2 mm). Its oxygen requirement can be satisfied by gassing the incubation fluid continuously with a 95% O_2 - 5% CO_2 mixture. Cannulation of the main pancreatic duct, which enters the duodenum separately from the common bile duct, permits collection of undiluted secretion from the ductal system.

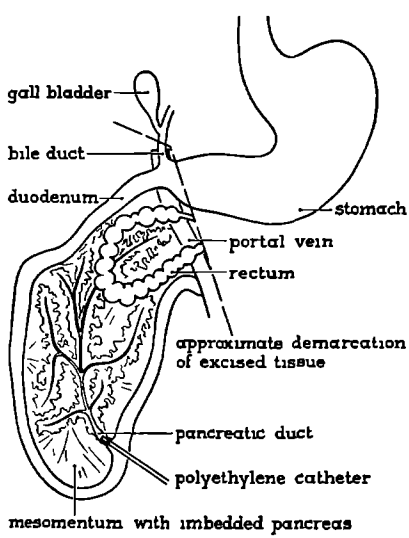


Fig 17 Anatomical topography of the rabbit pancreas

In vitro studies were carried out on the pancreas of male and female New Zealand white rabbits, weighing 2.0 to 3.0 kg. The animals were starved for 18 to 24 hr before each experiment and sacrificed by a sharp blow on the neck, immediately followed by carotic exsanguination. The pancreas was isolated in the following way: the attachments of other intestinal segments to the mesomental loop were cut, leaving a small loop of rectum attached to the omental surface in order to prevent damage to the omentum following removal of the loop. The mesomental loop was further isolated by cutting it away from the remaining intestine and from the mesentery, medial to the portal vein (Fig. 17). The isolated unit was removed and rapidly mounted on a polyvinyl chloride frame, similar to that previously described by Rothman (1964), by placing ligatures around various portions of the intestine wall and through appropriately spaced holes in the frame. The main pancreatic duct was then cannulated by means of polyethylene tubing (0 57 mm inner diameter). The frame was lowered into a plexiglas chamber (l x w x h = 20 x 2 x 10 cm) and the polyethylene catheter was passed through a hole in the chamber base. The isolated organ was bathed in 300 ml of a balanced salt solution. The bathing fluid was gassed continuously with 95% O_2 - 5% CO_2 and its temperature maintained at 30° C by pumping water of this temperature through the double-spaced wall of the chamber. The incubation solution was thoroughly mixed for the duration of each experiment by means of a magnetic stirrer and a magnetic bar, placed in the chamber. Approximately 10 min. were required to mount the isolated organ in the chamber after it had been removed from the animal.

2 BASAL PANCREATIC SECRETION

Pancreatic secretion was always collected under paraffin oil in small test tubes (8 mm inner diameter and 6 cm height) in hourly periods and the volume of the fluid was determined by weighing, assuming a specific gravity of 1.01, as determined by Maren (1956). Flow could not be expressed per unit tissue weight because of the difficulty in making accurate measurements of the proportion of pancreatic tissue present.

Without external stimulation the isolated rabbit pancreas secretes at rates up to 600 µl per hour, in agreement with previous findings of Rothman and Brooks (1965a). Rothman (1964) reported that the mean basal secretory rate in vivo in the fasted, anaesthetized rabbit over a four-hour period (excluding the first hour after cannulation) was 59,4 µl per hour, whereas the mean flow in the in vitro preparation taken from similarly fasted animals was 304 µl per hour over the same period This indicates that the isolated organ can produce pancreatic juice at flow rates about five times higher than in situ. This difference

may be due to a net inhibitory tone, nervous and/or hormonal, affecting the organ in vivo.

Table 6 shows the basal pancreatic fluid secretion during a total of four hours after mounting the isolated rabbit pancreas (n = 8).

Table 6

RATE OF BASAL PANCREATIC SECRETION FLOW AND ION CONCENTRATIONS

	1st hr	2nd hr	3rd hr	4th hr
Flow	299 ± 53.4	405 ± 46.0	500 ± 43.9	505 ± 49.3 *
Na^+	161 ± 2.2	169 ± 1.2	170 ± 1.4	171 ± 1.5
K^+	5.9 ± 0.4	6.8 ± 0.3	7.1 ± 0.3	7.5 ± 0.3
HCO_3^-	9.8 ± 1.6	9.5 ± 2.2	10.2 ± 3.5	10.5 ± 2.8
Cl^-	76.0 ± 2.1	87.2 ± 2.8	81.6 ± 2.8	79.1 ± 2.8

Time periods are hours 1-4 after mounting. Values for flow (in µl/hr) and for ion concentrations (in mmoles/liter) are given as means with standard errors for 8 experiments.

* Percent change relative to 3rd hr + 6 (SE 4.6) %

Flow increased for the first two hours after mounting and became relatively constant during the third and fourth hour. An average of 470 µl per hour was found during this four-hour period (excluding the first hour after mounting). This value is higher than the average flow of 304 µl per hour previously found by Rothman (1964) under the same experimental conditions.

The basal flow rate was unaffected by the hydrostatic pressure head. The chambers used in our experiments were 10 cm in height. If we assume the organ to be uniformly distributed over the height of the chamber, then there would normally be an effective average hydrostatic pressure of approximately five cm H_2O, favouring the outflow of fluid. When this pressure head was increased by placing the collecting tube five to ten cm above the chamber base, no change in flow and electrolyte secretion was observed, indicating that this change in the pressure head had no effect on outflow. This is in agreement with previous findings of Rothman and Brooks (1965a).

Flow remained relatively constant during both the third and the fourth hour after mounting (Table 6). Therefore, we used the third hour after mounting as a control period and the fourth hour as an experimental period in studying the effects of secretin, active transport inhibitors, acetazolamide, metabolic inhibitors and anaerobiosis on basal pancreatic fluid secretion in vitro.

Sodium and potassium in pancreatic fluid were determined by flame-photometry, while chloride determinations were carried out by means of the coulometric titration method of Cotlove et al (1961). Total CO_2 was measured in a Natelson microgasometer (Knight et al, 1957). Since the pH of pancreatic juice secreted by the rabbit pancreas in vitro is between 7 5 and 8 0 (Hubel, 1967), the values found for total CO_2 represent approximately the bicarbonate concentrations. For a more detailed description of the procedures used in ion determinations, reference should be made to chapter III.

Table 6 shows the mean values for flow and electrolyte concentrations during a four-hour period. In view of the rather drastic treatment in dissecting and mounting of the rabbit pancreas, we may consider the first hour of in vitro secretion as a recovery period and therefore leave it out of consideration During the three-hour period following the first hour after mounting the concentration of sodium remained relatively constant, while that of potassium showed a slight increase. The sum of alkali cation concentrations was 177 mmoles per liter (range 175 8 to 178.5 mmoles/liter) The concentration of bicarbonate on the other hand showed a slight increase and the chloride concentration a small decrease during the four-hour period The sum of both concentrations, however, remained constant (range 182 2 to 184 1 mmoles/liter and an average of 183 3 mmoles/liter). The difference between cationic and anionic osmolar concentrations is made up by Ca^{++} and Mg^{++}. The total osmolarity of the secreted fluid and the bathing fluid were equal.

The large bath volume relative to the secretory volume in our experiments left the concentrations of the various ions in the bathing fluid relatively constant throughout the experimental period Rothman and

Table 7

RELATION BETWEEN SODIUM CONCENTRATIONS OF BATHING FLUID AND SECRETORY FLUID

Bathing fluid	Secretory fluid 1st hr	2nd hr	3rd hr	4th hr	No of expts
150	146 ± 2 0	151 ± 1 5	152 ± 1 7	152 ± 2 3	3
170	161 ± 2 2	169 ± 1 2	170 ± 1 4	171 ± 1 5	8
195	196 ± 2 3	198 ± 1 1	198 ± 1 3	197 ± 1 6	6

Time periods are hours 1-4 after mounting Values for sodium concentration are expressed in mmoles/liter and are given as means with standard errors for respectively three, eight and six experiments

Brooks (1965a) demonstrated that under these conditions no changes are observed in the concentrations of sodium, bicarbonate and chloride of the bathing solution However, an increase in the concentration of potassium was observed by these authors within the first hour of secretion in vitro

Of interest is our observation that the concentration of sodium in pancreatic juice depends on the sodium concentration of the bathing fluid. When the sodium concentration of the standard balanced salt solution (170 mmoles/liter) was lowered to about 150 mmoles per liter or raised to 195 mmoles per liter, corresponding changes were found in the secretory sodium concentration (Table 7) The secretory sodium concentration in the various experiments always corresponded with the sodium concentration of the bathing solutions used. These results indicate that, as in the case of water reabsorption by the gall bladder (Diamond, 1962), we are dealing with an isotonic type of secretion in the pancreas

4 STIMULATION BY SECRETIN

Rothman and Brooks (1965a) showed that fluid secretion by the isolated rabbit pancreas is stimulated by a single dose of the hormone secretin (60 units/liter bathing fluid) to a small extent only (15%). The concentration of bicarbonate was not affected by secretin, in contrast with the increase in the concentration of bicarbonate after intravenous administration of secretin in different species (chapter I). Since the isolated rabbit pancreas is capable of producing five to eight times more fluid than in situ and is not influenced by the circulatory effects caused by secretin in vivo (Delaney and Grim, 1966), the isolated organ apparently secretes at a near-maximal rate and is therefore less sensitive to secretin.

Therefore, we decided to investigate the response to increasing doses of secretin. Secretin was added in four different doses to the bathing solution at the end of the third hour after mounting, which served as a control period. The flow rate during the control period was over 200 µl per hour. A maximal average flow stimulation of 35% (SE· 1 4) occurred after addition of 480 Crick-Harper-Raper units of secretin per liter bathing solution (about 57 6 units/kg body weight), while 60 units secretin per liter (about 18 units/kg) failed to affect flow rate (Table 8). Intravenous administration of the same doses of secretin in fasted animals would have caused a much higher stimulation of flow (Baxter, 1931) Stimulation of flow in vitro by secretin was not accompanied by a significant increase in the secretory bicarbonate concentration, in agreement with previous findings of Rothman and Brooks (1965a).

Table 8

EFFECT OF SECRETIN ON FLOW AND BICARBONATE CONCENTRATION

Secretin, units/1	Flow stimulation, %	Bicarbonate concentration, mmoles/1	
		control period	exptl. period
60	0	114 ± 16.8	114 ± 10.7
120	25 ± 1.3	94 ± 5.0	99 ± 3.6
240	30 ± 4.4	108 ± 2.5	115 ± 3.6
480	35 ± 1.4	107 ± 0.6	108 ± 3.6

At each concentration two experiments were performed.

5. EFFECT OF ACTIVE TRANSPORT INHIBITORS

In chapter IV we described the occurrence in dog pancreas of an adenosine triphosphatase, activated by sodium and potassium and inhibited by the cardiac glycoside ouabain, while in chapter V we demonstrated the inhibitory effect of ouabain on pancreatic fluid secretion in the dog. In chapter VI the occurrence of the $(Na^+\text{-}K^+)$-activated ATPase system in rabbit pancreas was described. The enzyme system was inhibited by several cardiac glycosides (chapter VI, section 3). Now the effect of cardiotonic steroids and the Erythrophleum alkaloid erythrophleine on water and electrolyte secretion by the isolated rabbit pancreas was investigated. Each cardiac glycoside, dissolved in either water or ethanol, was added to the bathing solution during the first 10 to 15 seconds of the fourth hourly collection period to give a final concentration of 10^{-5} M inhibitor. Erythrophleine was added in a 10^{-6} M concentration. Since digoxin, scillaren A and hexahydroscillaren A were dissolved in 80% ethanol, giving rise to a final ethanol concentration of 0.08% in the bathing solution, control experiments were carried out by adding the same volume of 80% ethanol to the bathing fluid.

The relative changes in the rate of flow, caused by ethanol, the four cardiac glycosides and erythrophleine are shown in Table 9. Ethanol (0.08%) had no effect on the flow rate. Ouabain (10^{-5} M), scillaren A (10^{-5} M) and digoxin (10^{-5} M) inhibited flow by 65, 60 and 35% respectively, indicating that the rabbit pancreas is apparently more sensitive to ouabain and scillaren A than to digoxin. Erythrophleine

Table 9

INHIBITION OF PANCREATIC FLUID SECRETION BY ACTIVE TRANSPORT INHIBITORS

Inhibitor	Concentration inhibitor	Flow inhibition, %	No of expts
Erythrophleine	10^{-6} M	44 ± 0 2	2
Ouabain	10^{-5} M	65 ± 1 3	4
Digoxin	10^{-5} M	35 ± 0 2	2
Scillaren A	10^{-5} M	60 ± 13 7	7
Hexahydroscillaren A	10^{-5} M	2 ± 3 7	2
Ethanol	0 08 %	4 ± 8 0	2

(10^{-6} M) inhibited flow rate by 44%, while hexahydroscillaren A (10^{-5} M), an inactive cardiac glycoside because of its saturated lactone group, did not alter pancreatic fluid secretion. The order of inhibitory effect is the same here as in the case of enzyme inhibition (Table 5, chapter VI), if one takes into account that erythrophleine was used in 10^{-6} M concentration only

Table 10 illustrates the effect of 10^{-5} M ouabain on both the flow and ion concentrations. An average flow inhibition of 65% was observed. The concentration of sodium remained relatively constant, while the potassium concentration showed a slight increase. The sum of alkali cation concentrations before addition of ouabain was 174.4 mmoles per liter and remained at about the same level after addition of ouabain (176.3 mmoles/liter). The bicarbonate concentration decreased slightly and the concentration of chloride increased a little, while the sum of both concentrations in the control and the experimental period remained constant (190 4 and 189 6 mmoles/liter respectively).

6 THE OUABAIN INHIBITION CURVE IN VITRO

In chapter V we reported that ouabain, injected directly into the arterial blood supply of the dog pancreas, inhibited the pancreatic outflow Unfortunately this approach does not allow calculation of the molar concentration of the injected cardiac glycoside on the active site and for this reason we could not compare the ouabain inhibition curve for the secretory system with that found for the enzyme system. Use of the isolated pancreas, however, enables us to know exactly the molar ouabain concentration applied to the secretory cells and there-

fore the ouabain inhibition curves for the secretory and (Na^+-K^+)-activated ATPase systems can be compared with each other.

Graded amounts of ouabain were added to the bathing medium at the beginning of the fourth hourly collection period to give final concentrations between 10^{-10} and 10^{-3} M ouabain. Fig. 18 shows that high

Table 10

EFFECT OF 10^{-5} M OUABAIN ON FLOW AND ION CONCENTRATIONS

	1st hr	2nd hr	3rd hr (control)	4th hr (ouabain)
Flow	565 ± 168.0	631 ± 148.7	719 ± 115.9	254 ± 31.9 *
Na^+	163 ± 2.8	168 ± 1.1	168 ± 1.5	168 ± 1.7
K^+	5.5 ± 0.3	6.0 ± 0.4	6.4 ± 0.3	8.3 ± 0.3
HCO_3^-	92 ± 6.2	101 ± 11.5	105 ± 9.0	95 ± 2.5
Cl^-	82.1 ± 2.1	86.4 ± 3.7	85.4 ± 6.9	94.6 ± 3.2

Time periods are hours 1-4 after mounting. Values for flow (in µl/hr) and ion concentrations (in mmoles/liter) are given as means with standard errors for 4 experiments. Ouabain was added to the bathing solution at the beginning of the fourth hourly collection period.

* Percent change relative to 3rd hr: -64.7 ± 1.3%.

concentrations of ouabain (> 10^{-8} M) inhibited pancreatic water secretion, while low concentrations (10^{-9} and 10^{-10} M), on the contrary, stimulated flow. At 10^{-3} M ouabain nearly complete flow inhibition (97%) occurred. The negative logarithm of the half-maximal inhibition concentration (pI_{50}) was 5.4, which is equal to the corresponding value for the (Na^+-K^+)-activated ATPase system (Fig. 15).

Both 10^{-9} and 10^{-10} M ouabain stimulated pancreatic fluid secretion. A maximal average flow stimulation of 19% (SE: 3.0) occurred at 10^{-9} M ouabain. For the (Na^+-K^+)-activated ATPase system a maximal stimulation of 16% (SE: 2.0) was observed at 10^{-9} M ouabain. So in the isolated rabbit pancreas ouabain has a biphasic effect on flow and the (Na^+-K^+)-activated ATPase system: inhibition at high concentrations and stimulation at low concentrations.

Similar biphasic effects have been observed in guinea-pig heart (Repke, 1963; Lee and Yu, 1963), in the kidney of chicken (Palmer and Nechay, 1964) and dog (Nahmod and Walser, 1966), in the toad bladder (McClane, 1965) and in cat choroid plexus (Oppelt and Palmer, 1966).

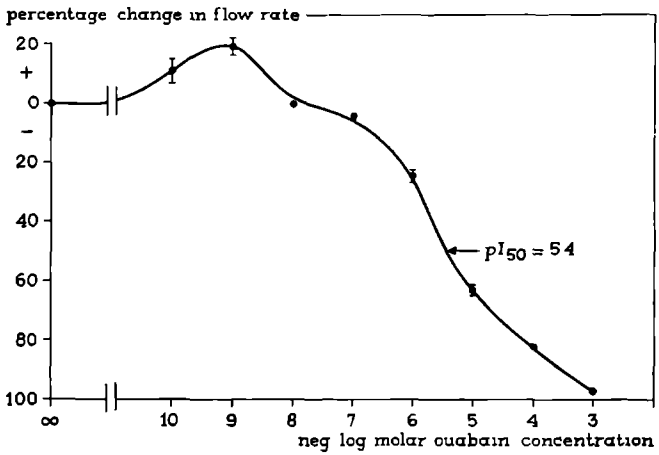


Fig 18 Effects of ouabain on fluid secretion by the isolated rabbit pancreas Graded amounts of ouabain were added to the bathing medium in final concentrations of 10^{-10} to 10^{-4} M at the beginning of the fourth hour after mounting The third hour after mounting was used as control period, the fourth hour as experimental period

7 EFFECT OF ACETAZOLAMIDE

Intravenous injection of 10 to 60 mg acetazolamide per kg body weight in the dog decreased in vivo pancreatic fluid secretion by approximately 50% (Birnbaum and Hollander, 1953, Rawls et al., 1963; Pak et al., 1966). However, under these conditions acid secretion by the kidney is also decreased due to inhibition of renal carbonic anhydrase activity (Maren et al., 1954). This results in a metabolic acidosis, which is known to inhibit pancreatic secretion in vivo in the dog (Rawls et al., 1963). Therefore, flow inhibition after acetazolamide in vivo is not solely due to a direct pancreatic effect. The isolated rabbit pancreas affords an ideal opportunity to study the direct effect of acetazolamide on exocrine pancreatic secretion, since fluid and electrolyte secretion in this case are not influenced by systemic effects.

Acetazolamide was added in graded amounts to the bathing solution at the end of the third hourly collection period (control period) with the next hour serving as the experimental period. Table 11 shows the effect of three different concentrations of acetazolamide on flow rate. At 0.3×10^{-3} M acetazolamide an average flow inhibition of 14% (SE 2.8) was observed. A maximal flow inhibition of 25% (SE· 3.3) was reached at 10^{-3} M acetazolamide, for 3×10^{-3} M did not give a significantly higher effect (27%, SE: 1.2, $P > 0.05$). The results in Table 12 show that the sodium concentration was not altered by acetazolamide, while the potassium concentration showed the usual slight increase. The bicarbonate concentration was slightly reduced and the

Table 11

INHIBITION OF PANCREATIC FLUID SECRETION IN VITRO BY ACETAZOLAMIDE

Acetazolamide concentration, moles/liter	Flow inhibition, %	No of expts
0.3×10^{-3}	14 ± 2.8	4
1.0×10^{-3}	25 ± 3.3	4
3.0×10^{-3}	27 ± 1.2	3

Acetazolamide was added at the end of the third hour to the bathing solution

chloride concentration was increased a little, while the sum of both concentrations remained relatively constant.

Table 13 shows the percentages flow inhibition for ouabain (10^{-6} M) and acetazolamide (10^{-3} M) separately and in combination Ouabain, in a concentration of 10^{-6} M, inhibited flow rate submaximally by 28% (SE: 1.5) Acetazolamide, in a 10^{-3} M concentration, inhibited flow rate maximally by 25% (SE 3.3) When ouabain (10^{-6} M) and acetazolamide (10^{-3} M) were added simultaneously to the bathing solution, a significantly increased flow inhibition of 43% (SE 3.2) was observed, indicating that the effect of these two drugs is additive.

Table 12

EFFECT OF 10^{-3} M ACETAZOLAMIDE ON FLOW AND ION CONCENTRATIONS

	1st hr	2nd hr	3rd hr (control)	4th hr (acetazolamide)
Flow	296 ± 56.4	413 ± 67.4	454 ± 52.1	339 ± 33.5 *
Na^+	163 ± 3.2	170 ± 1.4	171 ± 2.2	171 ± 2.6
K^+	5.6 ± 0.1	6.4 ± 0.2	7.0 ± 0.1	7.4 ± 0.2
HCO_3^-	9.7 ± 4.1	9.7 ± 2.5	9.9 ± 2.0	9.4 ± 3.7
Cl^-	79.3 ± 1.4	88.9 ± 3.7	86.4 ± 2.3	95.1 ± 1.6

Time periods are hours 1-4 after mounting Acetazolamide was added at the end of the third hour Values for flow (in µl/hr) and for ion concentrations (in mmoles/liter) are given as means with standard errors for four experiments

* Percent change relative to 3rd hr —25 ± 3.3%

Table 13

THE ADDITIVE EFFECT OF OUABAIN AND ACETAZOLAMIDE ON FLOW RATE

Inhibitor	Flow inhibition, %	No of expts
Ouabain (10^{-6} M)	28 ± 1 5	4
Acetazolamide (10^{-4} M)	25 ± 3 3	4
Combination	43 ± 3 2	3

8 EFFECT OF INHIBITION OF METABOLISM

Rothman (1964) demonstrated that fluid secretion by the isolated rabbit pancreas is affected by anaerobiosis. When the bathing fluid was gassed with a mixture containing 95% nitrogen and 5% carbon dioxide, a rapid decrease in flow rate was observed Virtually complete recovery of flow rate occurred when the organ was gassed again with the normally used 95% O_2 - 5% CO_2 mixture, indicating that the inhibition is reversible. Addition of sodium azide and sodium fluoride together to the bathing solution caused complete cessation of flow within 10 to 20 minutes. Since the inhibitor concentrations were not stated and the sodium concentration in the pancreatic juice was not determined in these experiments, we reinvestigated the effect of sodium azide and sodium fluoride on fluid and electrolyte secretion in vitro, but with some major differences. We studied the effects of sodium azide and sodium fluoride in final concentrations of 10^{-4} and 10^{-2} M on both flow and the concentration of sodium in pancreatic fluid. In these studies the effects on fluid secretion and secretory sodium were determined by comparing a control period (the third hour after mounting) to an experimental period (the fourth hour after mounting)

In order to study the effect of anaerobiosis on pancreatic fluid secretion and secretory sodium, the bathing solution was gassed with a gas mixture of 95% N_2 and 5% CO_2 during the fourth hourly collection period, which served as the experimental period. In other experiments sodium azide and sodium fluoride were added separately to the bathing fluid in final concentrations of 10^{-4} and 10^{-2} M and together in 10^{-4} M final concentration each. At the 10^{-4} M level sodium azide and sodium fluoride alone and in combination did not exert any influence on flow rate and sodium concentration, suggesting that Rothman used higher concentrations of these substances. This led us to test the effect of these inhibitors in a final concentration of 10^{-2} M.

Table 14 represents the effects of anaerobiosis, sodium azide (10^{-2} M)

and sodium fluoride (10^{-2} M) on flow and the sodium concentration in pancreatic juice. Fluid secretion appeared to be very sensitive to anaerobic conditions and to sodium azide, an inhibitor of cytochrome oxidase and consequently of oxidative phosphorylation. Gassing of the bathing fluid with a mixture containing 95% nitrogen and 5% carbon dioxide gave an average flow inhibition of 77% (SE 4.3), while 10^{-2} M sodium azide inhibited flow rate by 88% (SE 2.9). Sodium fluoride (10^{-2} M), an inhibitor of enolase and therefore of glycolysis, did not inhibit the flow. The concentration of sodium in pancreatic juice remained relatively constant in all three types of experiments. These findings indicate that the energy required for fluid and electrolyte secretion by the pancreas is mainly derived from oxidative phosphorylation.

Table 14

EFFECTS OF ANAEROBIOSIS AND METABOLIC INHIBITORS ON FLOW AND SECRETORY SODIUM

Agent		95% N_2 - 5% CO_2	Na-azide (10^{-2} M)	Na fluoride (10^{-2} M)
No. of expts		4	3	4
Flow	control period	421 ± 61.1	673 ± 79.7	434 ± 32.3
	exptl. period	102 ± 31.7	79.0 ± 1.2	490 ± 67.8
	percent change	77.1 ± 4.3	− 87.8 ± 2.9	+ 7.8 ± 12.5
Na^+	control period	174 ± 3.3	175 ± 1.3	168 ± 4.1
	exptl. period	180 ± 3.5	177 ± 0.7	171 ± 5.7

Values for flow (in µl/hr) and for sodium concentration (in mmoles/liter) are given as means with standard errors. For flow percent changes for each experiment were calculated, averaged and standard errors computed.

9. EFFECT OF A LOW SODIUM ENVIRONMENT

Incubation of the isolated rabbit pancreas in a salt solution, in which sodium was replaced partly by lithium, lowered fluid secretion severely (Rothman and Brooks, 1965b). The sodium concentration in the secreted fluid was also decreased considerably, possibly due to replacement by Li^+. In all experiments flow rate was restored on replacing the low sodium medium by the normal balanced salt solution, but recovery required at least two hours.

In view of the unknown role of lithium in these experiments, we carried out similar studies in which sucrose was added to the low sodium solution in order to maintain osmolarity. In a total of four experiments the standard salt solution, containing 170 mM Na^+, was replaced at

the end of the second hourly period by the modified salt solution, containing only 25 mM Na^+ (Table 2, chapter III) The average fluid secretion and cation concentrations are listed in Table 15 The hourly sodium output decreased 84%, from 88 to 14 μM Na^+, which is equal to the decrease of 85% in the sodium concentration of the bathing medium Although flow decreased rather dramatically in all four experiments (77%), no cessation of flow was observed This is in direct contrast to the results obtained by Rothman and Brooks (1965b), who observed a complete inhibition of flow in four out of five experiments in which they substituted a substantial part of the sodium for lithium. The concentration of sodium in the secreted fluid in our experiments showed a decrease from 164 to 130 mmoles per liter The concentration of potassium, on the contrary, increased from 8.0 to 20 7 mM K^+. Since the potassium concentration of the low sodium solution is about 10 mM (chapter III, section 2e), we must consider the possibility of an effect of potassium on fluid secretion. From the K^+-activation curve (Fig. 13) it is clear that 10 mM K^+ inhibits the Na^+-K^+ ATPase activity and thus the Na^+ pump by approximately 15% Flow, however, was inhibited by 77% upon incubation of the isolated organ in the low sodium environment. Moreover, Rothman and Brooks (1965a) observed an increase of the K^+ concentration in the normal medium during the experiment from 5 to 7 mM without observing an inhibitory effect on flow. Hence, an effect of the increased K^+ concentration on the flow in our experiments must be considered very unlikely. When the low sodium solution was replaced at the end of the third hourly collection period by the standard salt solution, flow rate recovered completely within one hour. The concentration of sodium increased and that of potassium returned to normal values, and both concentrations then remained relatively constant for the duration of the experiment. A fall in the sum of alkali cation concentrations from 172 to 151 mmoles per liter was observed during the one hour incubation in the low sodium solution. This decrease, however, was completely restored after replacement of the low sodium solution by the standard solution (169 7 mmoles/liter average during the three hours after replacement).

The difference between our results and those of Rothman and Brooks (1965b) may well be due to the presence of a high lithium concentration (119 mM) in their experiments. Since the cellular permeabilities of Li^- and Na^+ are similar in several biological systems (erythrocyte, Maizels 1954, frog skin Zerahn 1955, muscle, Keynes and Swan 1959), Li^+ apparently enters cells as readily as Na^+, but it cannot be actively secreted. For this reason lithium would accumulate in the secretory cells of the pancreas, and it would take considerable time before all intracellular Li^+ is replaced again by Na^+, explaining why recovery of pancreatic secretion lasted over two hours in the experiments of Rothman and Brooks (1965b) Our results indicate that fluid

Table 15

EFFECT OF A LOW SODIUM ENVIRONMENT ON FLOW AND CATION CONCENTRATIONS

	1st hr	2nd hr	3rd hr	4th hr	5th hr	6th hr
	170 mM Na^+		25 mM Na^+	170 mM Na^+		
Flow	412 ± 74.2	474 ± 67.1	110 ± 42.4	583 ± 64.9	585 ± 76.4	524 ± 81.2
Percent change			−77.3 ± 7.4	+18.3 ± 15.2		
Na^+	160 ± 1.7	164 ± 1.7	130 ± 4.1	154 ± 1.0	162 ± 1.7	164 ± 2.3
K^+	7.3 ± 0.3	8.0 ± 0.2	20.7 ± 0.8	10.9 ± 0.7	8.7 ± 0.4	8.7 ± 0.3

Time periods are hours 1-6 after mounting. Values for flow (in μl/hr) and cation concentrations (in mmoles/liter) are given as means with standard errors for 4 experiments. Sodium concentration in the bathing fluid was decreased to approximately 25 mmoles per liter during the third hour. Percent changes between 3rd and 2nd hr and between 4th and 2nd hr were calculated for each experiment, averaged and standard errors determined.

and electrolyte secretion in vitro are inhibited reversibly by a low sodium environment, such that the sodium output decreases proportionally to the sodium concentration in the bathing solution.

10 ^{22}Na SECRETION IN VITRO

Montgomery et al. (1941) first described the secretion of radioactive sodium by the pancreas in dogs, provided with pancreatic fistulae. After stimulation of pancreatic secretion by ingestion of lean meat, ^{22}Na was injected into the hind-leg vein of the animal. The radioactive isotope appeared in pancreatic fluid within the first three minutes after intravenous administration and maximum output of radioactivity occurred after 15 minutes.

In order to study the direct effect of ouabain and secretin on pancreatic sodium secretion we carried out similar experiments with ^{22}Na added to the bathing solution. The basal rate of ^{22}Na secretion by the isolated rabbit pancreas was determined by adding 20 μC ^{22}NaCl (specific activity > 1000 mC/g Na) to 300 ml bathing solution after preincubation for one hour. The secreted fluid was collected every 10 minutes during the next two hours. Radioactivity per 10-min period was measured in a scintillation counter (Philips PW 4119) Figs 19 and 20 show that under basal conditions, in all four control experiments radioactivity (total c p m /10-min period) increased during the first 60 min and then became relatively constant. Although radioactivity

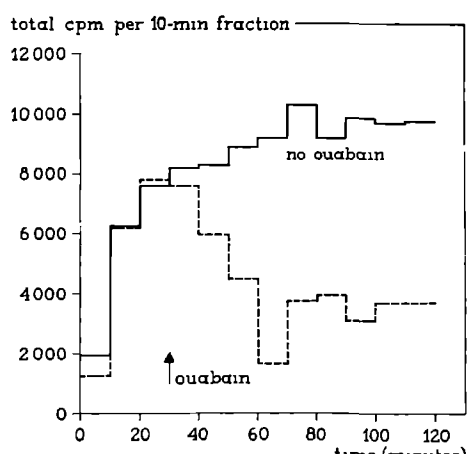


Fig 19 Inhibition by ouabain of the ^{22}Na secretion by the isolated rabbit pancreas Basal ^{22}Na secretion was determined by adding 20μC ^{22}NaCl to the bathing medium after a preincubation period of one hour Fluid was collected every 10 min and radioactivity per 10-min period was measured Ouabain (5×10^{-6} M) was added to the bathing fluid at the end of the third 10-min period

excreted per 10-min. fraction, like the flow rate varied with different preparations, it was relatively constant for a given preparation for at least the second hour after addition of the isotope to the incubation medium.

The effect of ouabain was determined by adding this cardiac glycoside to the bathing solution at the end of the third 10-min. period in a final concentration of 5 x 10^{-6} M. Compared to normal values (i.e. no ouabain added) an average inhibition of ^{22}Na secretion of 57% was observed (Fig. 19). This inhibition of ^{22}Na extrusion is in good agreement with the 58% flow inhibition and 50% inhibition of the (Na^+-K^+)-activated ATPase system found at this ouabain concentration (Figs. 18 and 15 respectively).

In order to answer the question whether secretin stimulates pancreatic secretion by a primary effect on Na^+ secretion, a similar type of experiment was carried out with secretin.

After a one-hour preincubation period 20 μC $^{22}NaCl$ was added to 300 ml bathing fluid. Then 480 Crick-Harper-Raper units per liter bathing solution were added at the end of the third 10-min. collection period. Fig. 20 shows that this dose of secretin stimulated ^{22}Na secretion by the isolated rabbit pancreas. Total radioactivity per 10-min. period increased during 20 minutes and then reached a maximum, representing an average stimulation of ^{22}Na secretion of 39%. This is approximately equal to the 35% stimulation of fluid secretion previously

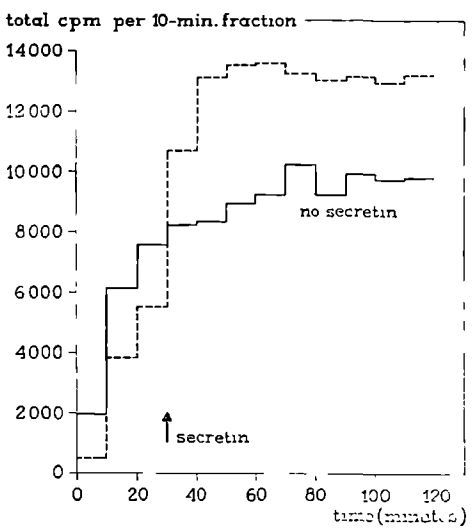


Fig. 20. Stimulation by secretin of the ^{22}Na secretion by the isolated rabbit pancreas. Basal ^{22}Na secretion was determined by adding 20μC $^{22}NaCl$ to the bathing medium after a preincubation period of one hour. Fluid was collected every 10 min. and radioactivity per 10-min. period was measured. Secretin (480 units/liter bathing fluid) was added to the bathing fluid at the end of the third 10-min. period.

found for the same dose of secretin (Table 8), suggesting that secretin stimulates the secretion of sodium by the pancreas and that the active secretion of sodium is the primary process in fluid and electrolyte secretion by this organ.

11 DISCUSSION AND CONCLUSIONS

The presence of the (Na^+-K^+)-activated ATPase system in the rabbit pancreas (chapter VI) and the similarity of its properties to those found for the enzyme system in other tissues, suggest that it might play a primary role in the secretion of fluid and electrolytes by the exocrine pancreas This is strongly supported by a number of experimental findings

(1) the inhibitory effect of cardiac glycosides and erythrophleine on the enzyme system as well as on pancreatic fluid secretion in the same order (Tables 5 and 9) and the identical pI_{50} for ouabain inhibition in both activities (5.4 and 5.4),

(2) ouabain in a 5×10^{-6} M final concentration inhibited pancreatic ^{22}Na secretion and flow rate to the same extent (inhibitions of 57 and 58% respectively),

(3) lowering the sodium concentration in the bathing solution decreased the sodium secretion proportionally, presumably by decreasing the sodium supply to the pump on the luminal side of the secretory cells,

(4) secretin (480 Crick-Harper-Raper units/liter bathing fluid) stimulated the secretion of radioactive sodium and flow rate to the same extent (39 and 35% respectively),

(5) ouabain in very low concentrations stimulated both the (Na^+-K^+)-activated ATPase system and fluid secretion; in both cases a maximal stimulation (19 and 16% respectively) was found at 10^{-9} M ouabain,

(6) hexahydroscillaren A, a cardiac glycoside with a saturated lactone group, did not significantly affect either pancreatic fluid secretion or (Na^+-K^+)-stimulated ATPase activity. Vates et al. (1964) made the same observation in the case of cerebrospinal fluid secretion, while Dunham and Glynn (1961) reported an analogous finding for active cation transport in erythrocytes. The requirement for an unsaturated lactone group for the inhibitory effect of cardiac glycosides on pancreatic secretion as well as on the (Na^+-K^+)-activated ATPase system, strongly supports a primary role of the latter system in pancreatic fluid and electrolyte secretion.

Active sodium transport by means of the (Na^+-K^+)-activated ATPase enzyme system has been shown to be the primary and rate-limiting event in the secretion of aqueous humour in the rabbit by Bonting and Becker (1964) and in the formation of cerebrospinal fluid in the cat by Vates and collaborators (1964). There are also indications

that the sodium pump plays an essential role in the secretion of sweat (Gibbs et al., 1967, Slegers, 1968) In these cases water appears to follow passively the actively secreted sodium For the gall bladder this has been demonstrated by Diamond (1962), who also showed that the mechanism involved is local osmosis (see chapter II, section 6) If in pancreatic secretion water also passively follows the actively secreted sodium, ouabain should not affect the concentration of sodium in pancreatic fluid, but only the total output of sodium in the juice This is indeed the case upon inhibition of flow by ouabain no change in sodium concentration was found in pancreatic secretion. Furthermore, ^{22}Na secretion by the isolated rabbit pancreas was inhibited by ouabain to the same extent as the rate of flow and secretin stimulated both modalities by nearly the same percentage These results indicate that the essential process in pancreatic fluid and electrolyte secretion is the active secretion of Na^+ rather than water secretion

Careful consideration of the results presented in this chapter leads to some further comments.

(a) When the isolated organ was incubated in a modified balanced salt solution, containing instead of the normal sodium concentration of 170 mmoles per liter, 150 or 195mM Na^+, secretory sodium concentration turned out to be approximately equal to the sodium concentration of the bathing fluid This means that we are dealing with an isotonic type of secretion, as previously found by Diamond (1962) for water reabsorption by the isolated gall bladder.

(b) The increase in potassium concentration in the presence of ouabain (Table 10) cannot be explained by a leakage of intracellular K^+ from the secretory cells into the duct due to inhibition of the sodium pump by ouabain. In that case the absolute amount of K^+ secreted should be higher after addition of ouabain than under control conditions. However, the results in Table 10 indicate a flow inhibition of 65% and an increase in K^+ concentration of only 30%

(c) Addition of acetazolamide to the bathing fluid gave an average maximal flow inhibition of 25% at a concentration of 10^{-3} M, which nearly completely inhibits the carbonic anhydrase enzyme system This shows that this enzyme cannot have a rate-limiting role in exocrine pancreatic secretion as previously proposed by Rawls et al (1963). The additive effect of ouabain and acetazolamide, as previously observed in the secretion of aqueous humour (Simon et al., 1962), suggests that ouabain and acetazolamide act on different systems within the pancreatic cells responsible for fluid and electrolyte secretion. Most likely acetazolamide affects the intracellular pH by inhibiting the carbonic anhydrase system and thus affects secretion secondarily (Slegers and Moons, 1968)

(d) Flow inhibition by anaerobiosis or by addition of Na-azide (10^{-2} M) a cytochrome oxidase inhibitor (Keilin and Hartree, 1939),

and the lack of inhibition by 10^{-2} M NaF, an enolase inhibitor (Warburg and Christian 1942), suggest that the secretory mechanism involved in pancreatic fluid and electrolyte secretion is highly dependent on oxidative energy, most probably derived from oxidative phosphorylation

(e) When the isolated organ was incubated in a salt solution containing a low sodium concentration, we observed in addition to the decrease in sodium output a decrease in the sodium concentration of the pancreatic fluid of 34 mM. Case et al. (1968) also reported a decrease in secretory sodium concentration of 30 mM after perfusion of the pancreas of the cat in situ with a solution containing 30 to 40 mM Na^+. Since in our experiments the total Na^- output decreased by the same percentage as the sodium concentration of the incubation fluid was decreased, it appears that at this low sodium concentration there occurs some back diffusion of Na^+ to the low Na^+ meduim.

In conclusion, the results presented here can be interpreted as follows: the ductular cells of the pancreas possess a considerable (Na^+-K^+)-stimulated ATPase activity, which acts as a sodium pump This pump system actively secretes Na^+ out of the cell. Water follows passively and isosmotically, most likely through local osmosis (Diamond, 1962). The sodium secretion should be the primary, rate-limiting process in exocrine pancreatic fluid and electrolyte secretion, as previously found for the secretion of aqueous humour (Bonting and Becker 1964) and the formation of cerebrospinal fluid (Vates et al., 1964).

Having thus reported our results and conclusions about the mechanism of pancreatic fluid and electrolyte secretion, we shall deal with the secretion of digestive enzymes by the pancreas in the next chapter.

THE MECHANISM OF PANCREATIC ENZYME SECRETION IN VITRO

INTRODUCTION

In the previous chapters attention was focused on only one aspect of exocrine pancreatic secretion, namely the secretion of fluid and electrolytes by the gland. The role of the (Na^+-K^+)-activated ATPase system, which is identical with or very closely related to the sodium pump, in pancreatic fluid secretion was investigated, both in vivo and in vitro. In chapters IV through VII the occurrence of an adenosine triphosphatase, activated by sodium and potassium and inhibitable by the cardiotonic steroid ouabain in both dog and rabbit pancreas was demonstrated. In the anaesthetized dog it was also shown that ouabain inhibits pancreatic fluid secretion without altering the concentrations of sodium and bicarbonate in the pancreatic fluid. A series of experiments with the isolated rabbit pancreas showed conclusively that this enzyme system plays an essential role in exocrine pancreatic fluid and electrolyte secretion. It was shown that the primary, rate-limiting step in this process is an active sodium secretion by means of an adenosine triphosphatase cation pump, which is activated by sodium and potassium. Water follows the actively secreted sodium passively and isotonically, presumably through local osmosis as in the case of the gall bladder (Diamond, 1962).

This chapter is concerned with the other aspect of exocrine pancreatic secretion, i.e. the secretion of digestive enzymes. With a continuous intravenous pancreozymin-secretin perfusion (0.5 Crick-Harper-Raper units/min. of each hormone) in the anaesthetized dog it was rather difficult to obtain a reasonably constant enzyme secretion. The unstimulated enzyme secretion (i.e. only secretin was perfused) was equally variable. In both cases enzyme secretion showed great variations between experiments as well as within a single experiment. In experiments with the isolated rabbit pancreas, however, basal enzyme secretion was relatively constant in an individual experiment although there was considerable variation between different preparations. Therefore, studies of the secretion of digestive enzymes were carried out with the isolated rabbit pancreas and the results are presented in this chapter.

The first section deals with a study undertaken to determine whether or not pancreatic enzyme secretion is related to the cation pump, the

primary and rate-limiting process in pancreatic fluid and electrolyte secretion Studies on the role of cyclic AMP, which apparently has an important function in the secretory mechanism of α-amylase by the parotid gland, are reported in the second section of this chapter.

1 THE ROLE OF THE SODIUM PUMP IN ENZYME SECRETION

In a variety of tissues the cation pump is coupled to the transport of various other substances, like sugars (Csáky, 1963a and 1963b) and amino acids (Csáky, 1963a and 1963b, Bittner and Heinz, 1963; Fox et al., 1964; Curran, 1965). Therefore, it was of interest to investigate whether the pancreatic sodium pump plays a role in the transport of digestive enzymes across the cell membrane.

We studied first the basal secretion of protein and α-amylase by the isolated rabbit pancreas. Once the pattern of secretion had been established, the effects of a variety of substances, including inhibitors of active cation transport and metabolism, on protein and α-amylase secretion in vitro were investigated in an attempt to determine whether cation transport and enzyme secretion in the pancreas are related.

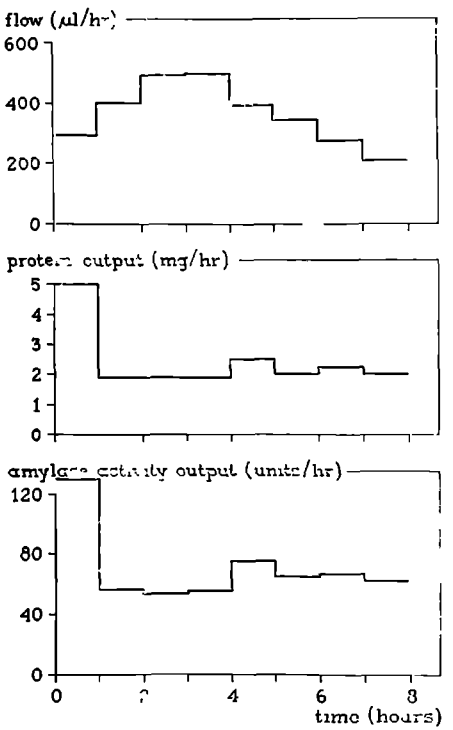


Fig 21 Basal fluid and enzyme secretion by the isolated rabbit pancreas Fluid was collected in hourly periods Protein and α-amylase activity were measured in the secreted fluid

a. *Basal pancreatic enzyme secretion*

In order to obtain an estimate of enzyme secretion by the isolated rabbit pancreas two parameters were used: total protein and α-amylase outputs The α-amylase activity in pancreatic juice was measured by determination of reducing sugars released from starch after a 3-min. incubation period with appropriate dilutions of pancreatic fluid at 30° C (Bernfeld, 1955). The hourly output of enzyme activity is expressed as units of activity secreted per hour. An activity unit is defined as one mg maltose liberated in 3 min by one ml of juice at 30° C. The protein content in pancreatic secretion was determined by the method of Lowry and associates (1951) with bovine albumin as a standard. The total hourly protein output, expressed as milligrams of protein secreted per hour, is an estimate of the total hourly output of digestive enzymes, including α-amylase. A detailed description of the procedures for determining α-amylase activity and the protein concentration in pancreatic secretion, is presented in chapter III. For the estimation of the total hourly outputs of α-amylase activity and protein, secretion was collected in hourly periods and the volume of the fluid was determined by weighing, assuming the specific gravity to be 1.01 (Maren, 1956).

Fig. 21 represents the basal secretion of fluid, protein and α-amylase from the isolated rabbit pancreas averaged for 13 experiments. In all cases secretion was collected during eight hourly periods whereafter experiments were terminated. Fluid secretion increased during the first two hours after mounting, remained relatively constant for the next two hours and then began to decrease. Protein and α-amylase secretion on the other hand were high in the first hourly collection period, but then decreased, remaining relatively constant during the next three hours and both increased somewhat during the next four hours, although fluid secretion in the same period tended to decrease. This is a first indication that the mechanism involved in enzyme secretion might be different from that responsible for fluid and electrolyte secretion Since the third and fourth hourly collection periods were relatively constant for both fluid secretion and the secretion of protein and α-amylase, they were used as control and experimental periods in studying the effects of several agents on fluid and enzyme secretion.

b *Effect of ouabain*

The cardiac glycoside ouabain in final concentrations varying between 10^{-7} and 10^{-3} M inhibits fluid secretion by the pancreas in vitro (chapter VII, section 6) Nearly complete flow inhibition was observed at 10^{-3} M ouabain (97%) Because the volume of pancreatic fluid after inhibition by respectively 10^{-3} and 10^{-4} M ouabain was rather small (10 to 60 µl/hr), the effect of 10^{-5} and 10^{-6} M ouabain on fluid secretion on the one hand, and on protein and α-amylase secretion on the

Table 16

EFFECT OF OUABAIN ON ENZYME SECRETION

Ouabain, moles/liter		Flow, µl/hr	Protein output mg/hr	Amylase output, units/hr	No of expts.
10^{-5}	control period	719 ± 115 9	2 0 ± 0 82	55 ± 15 5	3
	exptl period	254 ± 31 9	1 9 ± 0 65	50 ± 15 8	
	percent change	64 7 ± 1 3	+ 3 7 ± 14 9	- 10 4 ± 7 6	
10^{-6}	control period	681 ± 132 0	1 2 ± 0 07	38 ± 4 1	3
	exptl period	494 ± 99 1	1 1 ± 0 04	35 ± 5 8	
	percent change	- 27 7 ± 1 5	- 2 1 ± 7 2	- 8 0 ± 11 8	

Ouabain was added to the bathing solution at the beginning of the fourth hourly collection period Flow (in µl/hr), protein output (in mg/hr) and α-amylase activity output (in units/hr) are given as means ± SE for three experiments Percent changes between 4th (exptl period) and 3rd (control period) hr were calculated for each experiment, averaged and standard errors determined Only changes in flow were significant (P - 0 004)

other hand was investigated using the third and fourth hour after mounting as control and experimental period respectively.

Ouabain was added to the bathing solution at the beginning of the fourth hourly collection period to give final concentrations of 10^{-5} and 10^{-6} M inhibitor. Table 16 shows that although fluid secretion was inhibited by 65 and 28% respectively, the secretion of protein and α-amylase was not significantly affected by ouabain. Since ouabain is a specific inhibitor of the sodium pump involved in fluid and electrolyte secretion, this result is a second argument pleading against a coupling between cation and enzyme transport in the pancreas.

c. *Effect of acetazolamide*

Acetazolamide, a rather specific inhibitor of carbonic anhydrase, is capable of inhibiting fluid and electrolyte secretion in vitro (chapter VII, section 7). In contrast to ouabain which inhibited flow completely at 10^{-3} M concentration, the maximal inhibition by acetazolamide (10^{-3} - 3 x 10^{-3} M) was only 25-27% Combination of these drugs gave an additive effect, suggesting that ouabain and acetazolamide are acting on different systems in the pancreas

Acetazolamide was added in graded amounts to the bathing solution at the end of the third hourly collection period (control period) and secretion was studied during the next hour (experimental period). Table 17 shows the changes in flow rate caused by three different

Table 17

EFFECT OF ACETAZOLAMIDE ON ENZYME SECRETION

Acetazolamide, moles/liter		Flow, µl/hr	Protein output, mg/hr	Amylase output, units/hr	No of expts
0.3×10^{-1}	control period	434 ± 17.4	1.7 ± 0.31	47 ± 9.3	4
	exptl period	368 ± 11.3	1.8 ± 0.56	45 ± 12.8	
	percent change	14.2 ± 2.8	−0.5 ± 12.5	−2.5 ± 4.2	
1.0×10^{-3}	control period	454 ± 52.1	1.2 ± 0.42	43 ± 20.1	4
	exptl period	339 ± 33.5	1.4 ± 0.61	35 ± 15.6	
	percent change	24.6 ± 3.3	+6.9 ± 20.5	−17 ± 5.9	
3.0×10^{-3}	control period	463 ± 76.6	1.3 ± 0.23	24 ± 8.4	3
	exptl period	339 ± 64.6	1.1 ± 0.12	27 ± 7.4	
	percent change	26.9 ± 1.2	-21.4 ± 6.1	+2.8 ± 5.3	

Acetazolamide was added to the bathing fluid at the end of the third hourly collection period. Flow (in µl/hr), protein output (in mg/hr) and α-amylase activity output (in units/hr) are given as means ± SE for three or four experiments. Percent changes between 4th (exptl period) and 3rd (control period) hr were determined for each experiment, averaged and standard errors determined. Only changes in flow were significant ($P < 0.004$).

concentrations of acetazolamide and the effects of the same concentrations on protein and α-amylase secretion. A maximal average flow inhibition of 25% at 10^{-3} M inhibitor was observed. Protein and α-amylase secretion on the other hand were not affected by acetazolamide at these concentrations. These results suggest that carbonic anhydrase plays no role in enzyme secretion and plead also against a coupling of cation and enzyme transport.

d. *Effect of a low sodium environment*

In chapter VII (section 9) it was demonstrated that fluid and electrolyte secretion by the isolated rabbit pancreas is rather sensitive to changes in the sodium concentration of the bathing fluid. A marked inhibition of flow was observed after incubation of the isolated organ in a low sodium environment. Reduction of the sodium concentration in the bathing solution from 170 to 25 mmoles per liter Na^+ during the third hourly collection period, with sucrose added in order to maintain osmolarity, reduced the total Na^+ output by 84%. This reduction is

Table 18

EFFECT OF A LOW SODIUM ENVIRONMENT ON ENZYME SECRETION

	1st hr	2nd hr	3rd hr	4th hr	5th hr	6th hr	7th hr
	170 mM Na^+		25 mM Na^+	170 mM Na^+			
Flow	412 ± 74 2	474 ± 67.1	110 ± 42 4	583 ± 64.9	585 ± 76.4	524 ± 81.2	613 ± 99 1
percent change			— 77.3 ± 7 4 *	+ 18.3 ± 15.2			
Protein output	4 1 ± 0.82	1 1 ± 0 07	0.8 ± 0 14	2.5 ± 0.33	0 7 ± 0.7	0 5 ± 0.14	0 5 ± 0.10
percent change			— 23 ± 9 2	+ 137 ± 43			
α-amylase output	119 ± 13.1	31 ± 5.7	16 ± 1.7	59 ± 13.0	26 ± 11.3	16 ± 7.3	14 ± 5 2
percent change			— 46 ± 5 6 *	+ 86 ± 15 *			

Time periods are hours 1-7 after mounting. Flow (in μl/hr), protein output (in mg/hr) and α-amylase activity output (in units/hr) are given as means ± SE for four experiments. At the beginning of the third hour the bathing fluid with 170 mM Na^+ was replaced by a solution containing 25 mM Na^+ with sucrose added to maintain osmolarity. At the beginning of the 4th hour the original bathing fluid was substituted again. Percent changes between 3rd and 2nd hr and between 4th and 2nd hr were calculated for each experiment, averaged and standard errors determined. Significant changes (at $P = 0.05$ level) indicated with an asterisk.

equal to the decrease in the sodium concentration of the bathing solution, while flow on the other hand was decreased slightly less (77%)

In the same series of four experiments the total protein and α-amylase activity outputs were also determined. In the presence of a 25 mM sodium solution, protein secretion was not affected significantly, while the α-amylase activity output dropped by about 46%. Since the concentration of potassium of the low sodium solution is about 10 mM (chapter III, section 2e), we have to consider the possibility of an effect of potassium on enzyme secretion. There are no reports concerning the effect of high potassium concentrations on pancreatic enzyme secretion. High potassium concentrations, however, stimulate enzyme secretion by the parotid gland (Bdolah et al., 1964). In view of the functional resemblance between the parotid gland and the exocrine pancreas, we should expect 10 mM K^+ to stimulate pancreatic enzyme secretion. However, enzyme secretion was inhibited during incubation of the isolated organ in a low sodium environment. Hence, the decrease in the sodium concentration of the bathing solution must be responsible for the inhibition of pancreatic enzyme secretion. When the low sodium solution was removed at the end of the third hourly collection period and replaced by the standard balanced salt solution, flow returned to normal values and remained relatively constant for the duration of the experiment. This indicates that the flow inhibition produced by the low sodium environment is reversible. α-Amylase secretion however, showed a stimulation in the first hour after addition of the missing cation to the bathing fluid, but decreased again in the next three hours (Table 18). These findings constitute another indication that cation and enzyme transport in the pancreas are probably not coupled

e. *Effect of anaerobiosis and metabolic inhibitors*

In another attempt to determine whether or not cation and enzyme transport in the pancreas are related, we studied the effects of anaerobiosis and metabolic inhibitors on the secretion of digestive enzymes. Anaerobiosis was achieved by gassing the bathing solution with 95% N_2 - 5% CO_2 during the fourth hourly collection period. In other experiments sodium fluoride and sodium azide were added to the bathing fluid at the beginning of the fourth hourly collection period, to give final concentrations of 10^{-4} and 10^{-2} M inhibitor. Sodium fluoride and sodium azide in a final concentration of 10^{-4} M did not alter either fluid or enzyme secretion. A combination of the two substances in the same concentration also failed to affect both exocrine pancreatic fluid and enzyme secretion. For this reason a much higher concentration of sodium azide and sodium fluoride was used, i.e. 10^{-2} M of each salt. Table 19 shows the effects of nitrogen, sodium azide and sodium fluoride on the secretion of fluid, protein and α-amylase. Anae-

Table 19

EFFECTS OF ANAEROBIOSIS AND METABOLIC INHIBITORS ON ENZYME SECRETION

	Flow, µl/hr	Protein output, mg/hr	Amylase output, units/hr	No of expts
95% Nitrogen-5% CO_2				4
control period	421 ± 61 1	2 3 ± 0 80	55 ± 8 9	
exptl period	102 ± 31 7	1 0 ± 0 41	26 ± 10 4	
percent change	− 77 1 ± 4 3 *	57 ± 16 0 *	64 ± 11 9 *	
Na-azide (10^{-2} M)				3
control period	673 ± 79 7	1 2 ± 0 12	48 ± 16 3	
exptl period	79 ± 1 2	1 6 ± 0 31	42 ± 2 9	
percent change	− 87 8 ± 2 9 *	+ 40 ± 40	+ 24 ± 54	
NaF (10^{-2} M)				4
control period	434 ± 32 3	1 1 ± 0 21	44 ± 17 5	
exptl period	490 ± 67 8	9 1 ± 2 42	228 ± 79 5	
percent change	+ 7 8 ± 12 5	+ 744 ± 49 *	+ 441 ± 51 *	

The 3rd hour after mounting was used as control period, in the 4th hour the bathing fluid was gassed with 95% N_2 - 5% CO_2 or Na-azide and NaF were added to the bathing solution in a final concentration of 10^{-2} M at the beginning of the 4th hour, which served as experimental period Flow (in µl/hr), protein output (in mg/hr) and α-amylase activity output (in units/hr) are given as means ± SE for three or four experiments Percent changes between 4th and 3rd hr were calculated for each experiment, averaged and standard errors determined Significant changes (at P = 0 05 level) indicated with an asterisk

robiosis resulted in a flow inhibition of approximately 77%, while protein and α-amylase activity outputs were inhibited by 57 and 64% respectively. Sodium azide (10^{-2} M) gave an average flow inhibition of 88%, but failed to alter protein and α-amylase secretion significantly Sodium fluoride (10^{-2} M) on the other hand stimulated both protein and α-amylase outputs, but did not inhibit fluid secretion

These results indicate that the pancreas is highly dependent on oxidative energy for its exocrine function, but much less for the secretion of enzymes Again the results do not support a coupling of the Na^+ pump and enzyme secretion

f. *Discussion and conclusions*

The mechanism responsible for the release of digestive enzymes from zymogen granules across the cell membrane is not yet understood. Having found that pancreatic fluid and electrolyte secretion depends on an active sodium secretion by means of the ouabain-sensitive (Na^+-K^+)-activated ATPase system (chapters IV through VII), and knowing that in many instances other transport mechanisms (sugars and amino acids) are coupled to active sodium transport, the question arose whether enzyme secretion might be coupled to the sodium pump responsible for fluid and electrolyte secretion.

Of all the various parameters studied, not one gave indication of a coupling between the two secretory systems. The time course for basal protein and α-amylase secretion was different from that of fluid secretion (Fig 21). Ouabain in concentrations strongly inhibitory to flow did not alter enzyme secretion (Table 16). Acetazolamide showed similarly different effects on the two secretory systems (Table 17). Lowering of the sodium concentration in the bathing fluid reduced α-amylase output much less than Na^+ output and flow, while protein output was not influenced significantly (Table 18). About the same was true for anaerobiosis (Table 19). Sodium azide (10^{-2} M) strongly inhibited flow but did not reduce enzyme secretion Sodium fluoride (10^{-2} M) did not affect flow but stimulated enzyme secretion (Table 19) The latter effect might be due to complexing of Ca^{++}, leading to increased membrane permeability Another possible explanation is presented in the following section (subsection e).

These results constitute strong evidence that enzyme secretion in the pancreas is not coupled to the sodium pump, which plays a primary role in pancreatic fluid and electrolyte secretion. The fact that two different types of cells are responsible for these two types of secretion, the acinar cells for enzyme secretion and the ductular cells for fluid secretion (chapter I, sections 2 and 3), supports this conclusion

The question now arises, which mechanism is responsible for the secretion of enzymes by the pancreas Bdolah and Schramm (1965) demonstrated that cyclic AMP plays an important role in α-amylase secretion by parotid gland slices. Because the exocrine pancreas and the parotid gland have several properties in common, we decided to investigate the possible role of cyclic AMP in pancreatic enzyme secretion. Results of this study are given in the next section

2 CYCLIC AMP AND PANCREATIC ENZYME SECRETION

The enzymes secreted by the acinar cells of the pancreas, are derived to a major extent from the zymogen granules (Palade et al., 1962). Electron microscopy studies indicate that the release of digestive enzymes occurs by means of a reversed pinocytosis· the zymogen gra-

nule attaches to the cell membrane and after opening of the membrane the granular contents are released into the lumen. Inducers of pancreatic enzyme secretion such as pancreozymin and cholinergic agents, must at some point in this sequence initiate or accelerate this process. Unfortunately, there is almost no information on the biochemical reactions underlying this most important phase of enzyme secretion.

Since the discovery of cyclic AMP (Sutherland and Rall, 1960), a number of hormones has been shown to exert their influence on their target tissues by an alteration of the steady-state level of cyclic AMP in the cell (Sutherland et al., 1968). There are two enzymes known to play a part in the control of the intracellular levels of cyclic AMP. Adenyl cyclase, an enzyme localized in the cell membrane, catalyses the conversion of ATP to cyclic AMP (Davoren and Sutherland, 1963). A variety of hormones can stimulate this enzyme, both in homogenates and in the intact cell. The other enzyme which controls the intracellular cyclic AMP levels, is a phosphodiesterase. This enzyme, which is located partly in the particular material and partly in the soluble fraction of the cell, hydrolyzes cyclic AMP to AMP (de Robertis et al., 1967; Cheung, 1967). Several drugs, including the methylxanthines (Butcher and Sutherland, 1962), certain benzothiadiazine derivatives (Schultz et al., 1966) and puromycin (Appleman and Kemp, 1966) inhibit the phosphodiesterase. Among all the physiological processes mediated by cyclic AMP, the secretion of macromolecules by various endocrine and exocrine glands (Bdolah and Schramm, 1965; Babad et al., 1967; Turtle et al., 1967; Scholefield, 1967) forms an important group.

Of particular interest to investigators studying the exocrine pancreas is the observation of Bdolah and Schramm (1965) that cyclic AMP is an intermediate in the stimulation of parotid gland secretion by epinephrine. Inhibition of the phosphodiesterase by 1,3-dimethylxanthine in parotid slices enhanced α-amylase secretion into the incubation medium, presumably by raising the steady-state level of cyclic AMP within the cell up to concentrations required to cause a discharge of α-amylase. Cyclic AMP, up to a concentration of 9 mM, failed to stimulate significantly the secretion of α-amylase This is apparently due to the fact that the parotid gland cells are impermeable to the nucleotide. Many other tissues seem to be rather impermeable to cyclic AMP (Rall and Sutherland, 1962). Dibutyryl cyclic AMP, however, was shown to be a potent inducer of enzyme secretion by parotid gland slices, even in a concentration of 1 mM.

In view of the morphological and functional resemblance between the parotid gland and the exocrine pancreas, it seemed likely that enzyme secretion by the pancreas would also be mediated by cyclic AMP. These considerations led us to investigate the role of cyclic AMP in enzyme secretion by the isolated rabbit pancreas.

a *Stimulation of enzyme secretion*

In studying the effects of various agents on enzyme secretion by the isolated rabbit pancreas, the third and fourth hour after mounting were again used as control and experimental period because of the relative constancy of secretion during these periods

Addition of the duodenal hormone pancreozymin to the bathing solution at the beginning of the fourth hourly collection period always caused a massive release of both protein and α-amylase into pan-

Table 20

STIMULATION OF ENZYME SECRETION BY PANCREOZYMIN

Pancreozymin, units/liter	Stimulation	
	Protein secretion	Amylase secretion
	x control level	x control level
8 5	3 1	2 2
17 0	5 4	3 5
34 0	6 2	4 2
68 0	9 7	6 3
136 0	13 4	6 4

The third hour after mounting was used as control period, pancreozymin was added at the beginning of the fourth hour, which served as experimental period Averages for duplicate determinations

creatic juice. Table 20 shows the results obtained for five different doses of pancreozymin on the secretion of protein and α-amylase. A maximal stimulation of protein secretion was observed after 136 Crick-Harper-Raper units per liter bathing fluid, while α-amylase secretion was already maximally stimulated by 68 units of pancreozymin per liter bathing solution.

Addition of acetylcholine in final concentrations of 10^{-6} to 10^{-4} M, also resulted in a large discharge of protein and α-amylase from the acinar cells into pancreatic juice (Table 21) There was a maximal stimulation for both protein and α-amylase secretion at 10^{-4} M acetylcholine.

These results clearly indicate that enzyme secretion from the isolated rabbit pancreas is sensitive to pancreozymin and acetylcholine, in agreement with findings of Rothman (1967). However, both agents, in all doses used, stimulated protein secretion somewhat more than α-amylase secretion.

Table 21

STIMULATION OF ENZYME SECRETION BY ACETYLCHOLINE

Acetylcholine, moles/liter	Stimulation	
	Protein secretion	Amylase secretion
	x control level	x control level
10^{-6}	5 1	3 1
10^{-5}	10 7	4 2
10^{-4}	16 5	5 6

Conditions same as in Table 20 with acetylcholine replacing pancreozymin

b. *Effects of methylxanthines*

The effects of 1,3-dimethylxanthine (theophylline) and 3,7-dimethylxanthine (theobromine), on protein and α-amylase secretion, were studied after addition of each of the methylxanthines to the incubation fluid at the end of the third hourly collection period in a final concentration of 10^{-2} M. Both protein and α-amylase secretion were stimulated by 10^{-2} M theophylline (Fig 22). During the fourth hour an average stimulation for α-amylase secretion of 46% was observed, while protein secretion was stimulated by 82%. A maximal average stimulation for α-amylase secretion of 80% was found during the third hour after addition of the drug. Protein secretion was maximally stimulated during the second hour after theophylline addition by approximately 155%. Theobromine, in the same concentration as theophylline

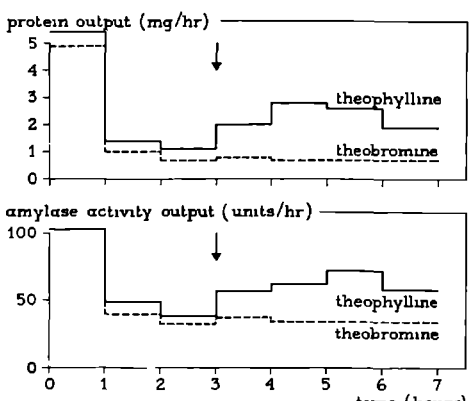


Fig 22 Effects of methylxanthines on enzyme secretion by the isolated rabbit pancreas Fluid was collected in hourly periods Protein and α-amylase activity were measured in the secreted fluid Theophylline and theobromine, both in 10^{-2} M final concentration, were added to the bathing solution at the end of the third hour after mounting

(10^{-2} M), had little effect on protein and α-amylase secretion from the isolated rabbit pancreas (about 5 to 10% stimulation). This is in agreement with previous results obtained by Butcher and Sutherland (1962) who observed that theophylline is an 8-10 times more potent inhibitor of a partially purified 3', 5' nucleotide phosphodiesterase preparation than theobromine. Fig. 23 shows that 10^{-3} M theophylline exerted about the same small effect on protein and α-amylase secretion in vitro (about 5 to 10% stimulation) as 10^{-2} M theobromine.

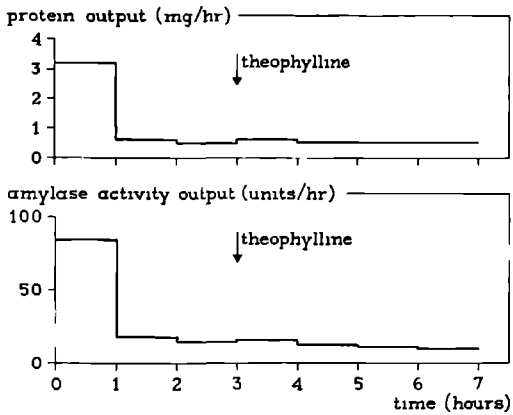


Fig 23 Effect of theophylline on enzyme secretion by the isolated rabbit pancreas Fluid was collected in hourly periods Protein and α-amylase activity were measured in the secreted fluid Theophylline (10^{-3} M final concentration) was added to the bathing fluid at the end of the third hour after mounting

c. *Effect of cyclic AMP*

The effects of two concentrations of cyclic AMP (10^{-4} and 10^{-3} M) on protein and α-amylase secretion were investigated. The nucleotide was added to the bathing solution at the end of the third hourly collection period and enzyme secretion was studied during the next four hours. In a concentration of 10^{-4} M, cyclic AMP failed to stimulate both protein and α-amylase secretion (Fig. 24). In a concentration of 10^{-3} M, cyclic AMP caused an average maximal stimulation for protein secretion of 64% two hours after addition of the nucleotide, while α-amylase secretion was maximally stimulated by approximately 31%, three hours after addition of cyclic AMP to the bathing solution (Fig. 24).

These findings indicate that cyclic AMP, added to the incubation fluid, is able to mimic the effect of pancreozymin on pancreatic enzyme secretion. Cyclic AMP, like pancreozymin and theophylline, stimulated total protein secretion to a greater extent than α-amylase secretion in vitro The results in Fig 24 also show that the rate of enzyme secretion increased with time, indicating that cyclic AMP very slowly penetrates pancreatic cells.

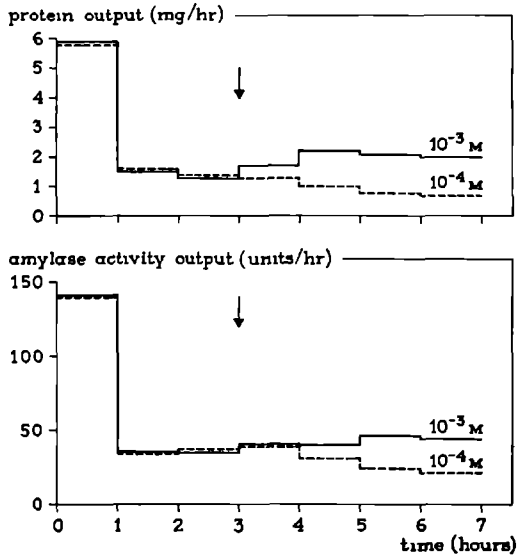


Fig 24 Effect of cyclic AMP on enzyme secretion by the isolated rabbit pancreas Fluid was collected in hourly periods Protein and α-amylase activity were measured in the secreted fluid Cyclic AMP (10^{-4} and 10^{-3} M final concentrations) was added to the bathing fluid at the end of the third hour after mounting

d. *The combined effect of pancreozymin and theophylline*

If pancreozymin enhances secretion by stimulating the adenyl cyclase, which is located in the cell membrane and catalyses the formation of cyclic AMP from ATP, then its action on protein secretion should be potentiated by drugs which inhibit the phosphodiesterase, which hydrolyzes cyclic AMP. This statement is obviously subject to some qualifications. It is necessary to ascertain that the effect of phosphodiesterase inhibition is not being obscured by some other effect of the drug. Dose levels are critical too in attempting to satisfy this criterion If the concentration of the hormone is such that the level of cyclic AMP is already high enough to produce a maximal response, then inhibition of the phosphodiesterase activity will not cause a further increase in the response In our study we used the lowest dose of pancreozymin (8 5 Crick-Harper-Raper units/liter bathing fluid), which still was able to produce a large discharge of enzymes by the isolated rabbit pancreas (Table 20), and 10^{-2} M theophylline.

Both pancreozymin (8 5 units/liter fluid) and theophylline (10^{-2} M) were added to the bathing solution at the end of the third hourly collection period and the third hour was used as control period Pancreozymin alone stimulated protein secretion by 211%, while α-amylase secretion was stimulated by 120% (Table 22) Theophylline alone

Table 22

EFFECT OF THEOPHYLLINE ON THE ACTION OF PANCREOZYMIN

	Stimulation %	
	Protein secretion	Amylase secretion
Pancreozymin (8 5 units/liter)	211 ± 11	120 ± 13
Theophylline (10^{-2} M)	82 ± 2 3	46 ± 2 9
Combination	493 ± 10	194 ± 14

The third hour after mounting was used as control period pancreozymin was added at the beginning of the fourth hour, which served as experimental period, pancreozymin in combination with theophylline was added at the end of the third hour All values for stimulation are given as means ± SE for four experiments

stimulated protein secretion by 82%, while α-amylase secretion was stimulated by 46% (Fig. 22). In combination with theophylline, however, pancreozymin caused a 493% stimulation of protein secretion. For the secretion of α-amylase a 194% stimulation was observed. Thus it can be stated that theophylline potentiates the effect of pancreozymin.

e *Discussion and conclusions*

Cyclic AMP was initially discovered as the intracellular mediator of the glycogenolytic effect of epinephrine and glucagon on the liver (Sutherland and Rall, 1960). Since then it has been recognized as a mediator in a variety of hormonal effects (Sutherland et al., 1965; Sutherland and Robison, 1966, Orloff and Handler, 1967; Robison et al., 1967) In all these cases the hormones seem to act by stimulating the adenyl cyclase, the enzyme that catalyses the formation of cyclic AMP from ATP and thus controls the intracellular level of cyclic AMP

In the case of the stimulation of pancreatic enzyme secretion by pancreozymin we demonstrated a stimulating effect of exogenous cyclic AMP (10^{-3} M). The presence in the pancreas of 3′, 5′ nucleotide phosphodiesterase, which hydrolyzes cyclic AMP, has been reported (Butcher and Sutherland, 1962). We could also demonstrate significant stimulation of enzyme secretion (46 to 155% depending on time) after addition to the bathing fluid of theophylline (10^{-2} M), which inhibits this enzyme Theophylline at 10^{-3} M and theobromine at 10^{-2} M had the same small effect (about 5 to 10% stimulation), which is in agreement with the relative potency of these substances towards the enzyme (Butcher and Sutherland, 1962). Simultaneous addition of pancreo-

zymin in a dose stimulating submaximally and of theophylline (10^{-2} M) caused a potentiating effect. Finally it was observed that pancreozymin, cyclic AMP and theophylline all stimulated protein secretion to a larger degree than α-amylase secretion. Although we did not determine the effect of pancreozymin on adenyl cyclase activity or the cyclic AMP level in pancreatic tissue, the above-mentioned evidence strongly indicates that cyclic AMP mediates the effect of pancreozymin on pancreatic enzyme secretion.

Upon completion of these experiments a report by Kulka and Sternlicht (1968) appeared, in which similar studies on enzyme secretion in mouse pancreas in vitro are described. They find theophylline, cyclic AMP and its mono- and dibutyryl derivatives to be potent stimulators of α-amylase secretion, but not of the synthesis of this enzyme. Their conclusion is, like ours, that pancreatic enzyme secretion is mediated by cyclic AMP. How the latter substance stimulates enzyme secretion is still unknown. Some indication is given by the observation of Aulich et al. (1967) that it activates phospholipase activity in isolated fat cells Since phospholipase occurs in an inactive form in the pancreas (de Haas et al., 1968), cyclic AMP might activate pancreatic phospholipase, as it does in the case of phosphorylase in the liver (Sutherland and Rall, 1960). The activated enzyme would attack the phospholipids of the plasma membrane, giving rise to the formation of lysophosphatides, which are known for their lytic effect on membranes (Gottfried and Rapport, 1963). The lysophosphatides might be formed at the point of fusion of the zymogen granule and the plasma membrane of the acinar cell, thus leading to enzyme release. This hypothesis concerning the role of cyclic AMP in pancreatic enzyme secretion is illustrated in Fig. 25.

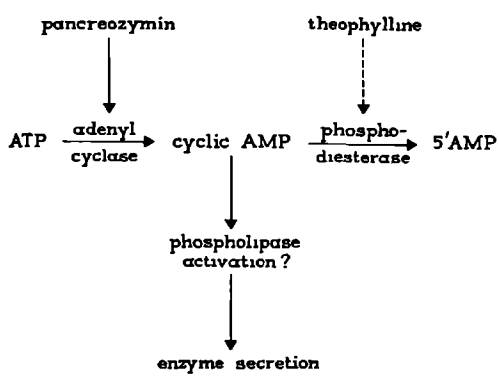


Fig 25. Schematic diagram of the role of cyclic AMP in pancreatic enzyme secretion.

Two final comments are in order. The larger stimulation of protein secretion than α-amylase secretion suggests that the secretion of different enzymes is stimulated to a different extent by pancreozymin and the other agents. Rothman (1967) has reported that pancreozymin and methacholine stimulate the release of trypsin, but not that of chymotrypsin. The reason for this differential stimulation is not yet clear The second point concerns the effect of NaF on enzyme secretion The stimulation of enzyme secretion by NaF (10^{-2} M) reported in section 1 (subsection e) of this chapter may be explained by the stimulating effect of this substance on adenyl cyclase, as previously showed in various tissue homogenates (Sutherland et al , 1962; Øye and Sutherland, 1966).

AN ELECTRONMICROSCOPIC STUDY OF THE FLUID TRANSPORT SYSTEM IN THE PANCREAS

INTRODUCTION

Studies of solute and water transport in the fish and rabbit gall bladder (Diamond, 1962; Wheeler 1963) have revealed that active sodium transport creates the driving force for the absorption of water from the gall bladder lumen, and that water absorption may even proceed against significant osmotic gradients.

In a combined physiological and electronmicroscopic study, Kaye et al. (1966) have demonstrated that in actively reabsorbing gall bladders, either in vivo or in vitro, the epithelial intercellular spaces are always distended. When sodium was omitted from the bathing solution, fluid transport was inhibited and no distention of these intercellular spaces was observed Addition of the missing cation rapidly led to the re-establishment of fluid transport and corresponding distention of the epithelial intercellular spaces. They also found that the distended intercellular spaces were always closed by means of junctional complexes at the luminal side, while narrow openings were observed at the serosal side. This observation indicates that fluid is transported in the direction of the capillaries located in the serosa. Studies of sodium localization by fixation with a pyroantimonate-OsO_4 mixture showed high concentrations of this ion in the distended intercellular spaces.

From the literature (chapter I) it is known that most likely the ductular cells are the site of production of a plasma-like salt solution by the pancreas. In this thesis evidence has been presented, showing that active sodium transport is the primary and rate-limiting process in exocrine pancreatic fluid and electrolyte secretion. The question now arises whether or not similar ultrastructural alterations as in the active gall bladder occur in the exocrine pancreas, when it is secreting fluid and electrolytes, and which cells are involved in this process.

In order to answer these questions, an electronmicroscopic study of the resting and secreting pancreas was undertaken.

1 MORPHOLOGICAL ASPECTS OF PANCREATIC FLUID SECRETION

a. *Preparation of animals*

The pancreas of the dog was studied before and after secretin administration. The animals were starved for 24 hours before each

experiment and they were initially anaesthetized with 15 to 20 mg sodium thiopentone per kg body weight, injected intravenously. Subsequently a tube was placed in the trachea and connected to a semi-closed system provided with a CO_2 absorber and a halothane vapourizer. By means of this system controlled amounts of N_2O (2 liter/min.), oxygen (1 liter/min.) and halothane (2-3%) were introduced into the trachea to keep the animals in early stage three of anaesthesia The abdomen was opened through a midline incision. Specimens of pancreatic tissue (biopsies) were taken, after which secretin (1 unit/kg body weight) was injected into the left femoral vein. After secretin administration specimens of tissue were taken at various intervals.

b. *Fixation and embedding*

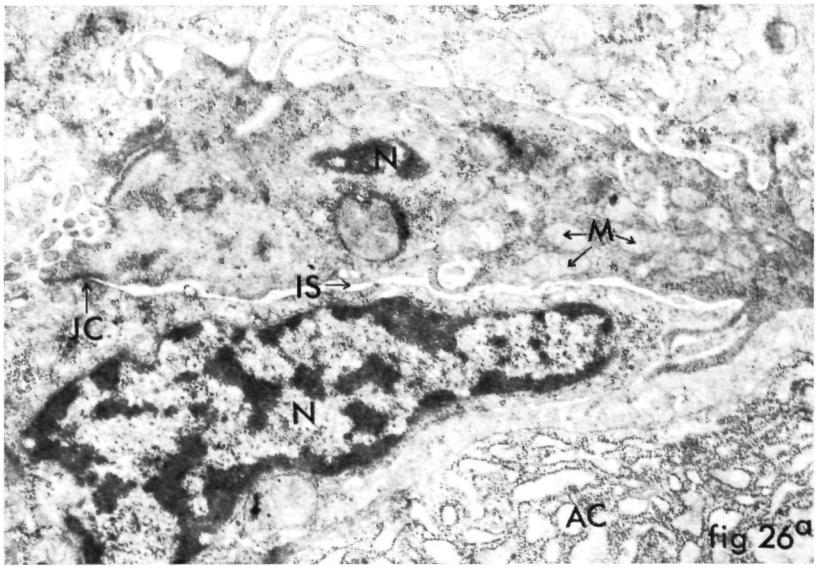
After immediate prefixation with chilled 3% glutaraldehyde in cacodylate buffer (pH 7.3), the biopsies were post-fixed with 2% OsO_4 in veronal/acetate buffer (pH 7.2-7.4), dehydrated in a graded series of ethanol solutions and embedded in Epon 812 (Luft, 1961).

Thin sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate (Reynolds, 1963) and studied in a Philips EM 300 electron microscope.

c. *The ultrastructure of the ductular cells*

We first studied thin sections prepared from biopsies taken before secretin administration. Survey electron micrographs showed that the ductular cells are considerably smaller than the acinar cells, in agreement with earlier findings of Ekholm et al. (1962).

The acinoductular cells, i.e. the ductular cells closest to the acinar cells, were elongated The nucleus also had an elongated shape with a highly irregular nuclear outline. The sparse cytoplasm was of low over-all electron density (Fig. 26a), and contained little endoplasmic reticulum. Only occasionally very short ergastoplasmic membranes and a few round membrane profiles with scattered ribosomes were observed. Free ribosomes, generally clustered into small groups, were more numerous, but still far less than in the acinar cells. Mitochondria were present in considerable numbers in the acinoductular cells. These organelles had a normal ultrastructure and septate cristae, transversly orientated. In nearly all cells a rather modest Golgi complex, consisting of a few paired membranes, vacuoles and vesicles, was found. In addition, in all acinoductular cells the presence of one body of moderate and inhomogeneous electron density and of varying size was noted (Fig. 27). To our knowledge the existence of such a "lipoid body" has not yet been reported for the acinoductular cells of other species. The intercellular space between two adjacent acinoductular cells and that between an acinoductular and an acinar cell were not distended in the resting pancreas (Fig. 26a) These spaces were closed at one side by



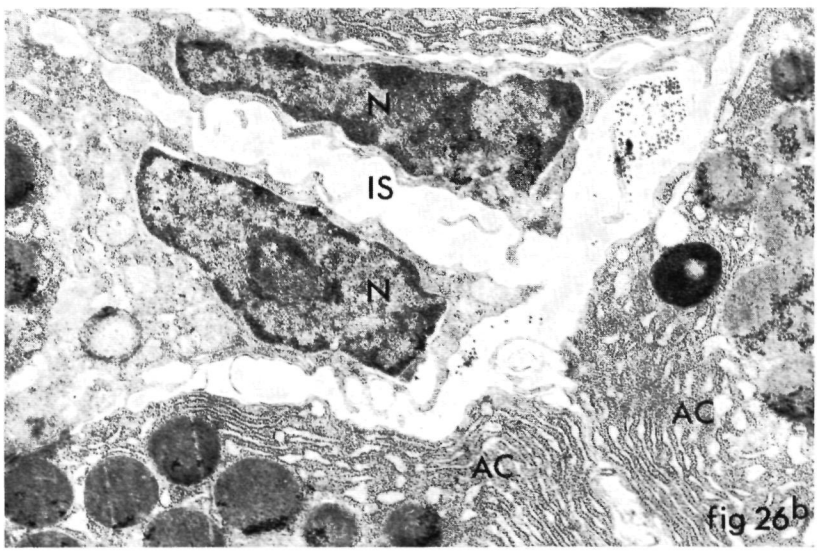


Fig. 26. a: Acinoductular cells from resting dog pancreas. The nucleus (N) occupies the major part of the cell. Mitochondria (M) are present in the cytoplasm. The intercellular space (IS) between the acinoductular cells is closed at one side by means of a junctional complex (JC) and open at the opposite side. x 21,000. b: Acinoductular cells from secreting pancreas. No changes are noted in the ultrastructure of the cells. The intercellular space (IS) between two acinoductular cells is enlarged. x 35,000.

means of a tight junction, while the opposite side was open via a rather narrow opening (140 to 400 Å).

The intercalary and intralobular ductular cells possessed an ultrastructural organisation very similar to that of the acinoductular cells. Their nuclei had marginal indentations. The cytoplasm of the ductular cells was equally sparse and poor in elements belonging to the endoplasmic reticulum. A very small number of short granular membranes was observed. Free ribosomes, usually aggregated in small groups, were more numerous than those attached to membranes. A considerable number of mitochondria was present. The Golgi complex had modest dimensions. Most of the ductular cells also possessed a lipoid body as present in the acinoductular cells (Fig. 28). The intercellular spaces between the ductular cells were not distended and they were always closed at the luminal side by means of tight junctions and open at the basement membrane side. The lumen was filled with a rather dense material. At the luminal side of the ductular cells cilium-like structures were observed. The ductular cells merely differ from the acinoductular cells in their shape, for they appear cuboidal or short columnar in survey electron micrographs.

d. *Ultrastructural changes following secretin stimulation*

Thin sections prepared from biopsies taken after secretin administration were studied next. The acinoductular cells showed no striking changes in their ultrastructure after secretin stimulation. The only striking change commonly observed was a distention of the intercellular spaces between adjacent acinoductular cells and between acinoductular and acinar cells (Fig. 26b). Moreover, the lateral plasma membranes now demonstrated a large number of microvilli, which might indicate that changes in membrane activity occur after secretin stimulation. The intercellular spaces were always completely transparent to electrons.

The ductular cells demonstrated the same major changes, i.e. distention of the intercellular spaces and pronounced irregularities of the lateral plasma membrane (Fig. 28b). Moreover the ductular lumen was enlarged. Sometimes the lumen contained varying amounts of cytoplasmic material, apparently derived from the acinar cells which were strongly stimulated by pancreozymin present in the secretin preparation used. Again the intercellular spaces were closed at the luminal side by means of a typical junctional complex, while at the basement membrane side there was a narrow opening (about 500Å).

2. DISCUSSION AND CONCLUSIONS

The results presented in this chapter support the opinion of Ekholm and associates (1962) that the acinoductular cells and the cells lining the intercalary and intralobular ductules show great ultrastructural

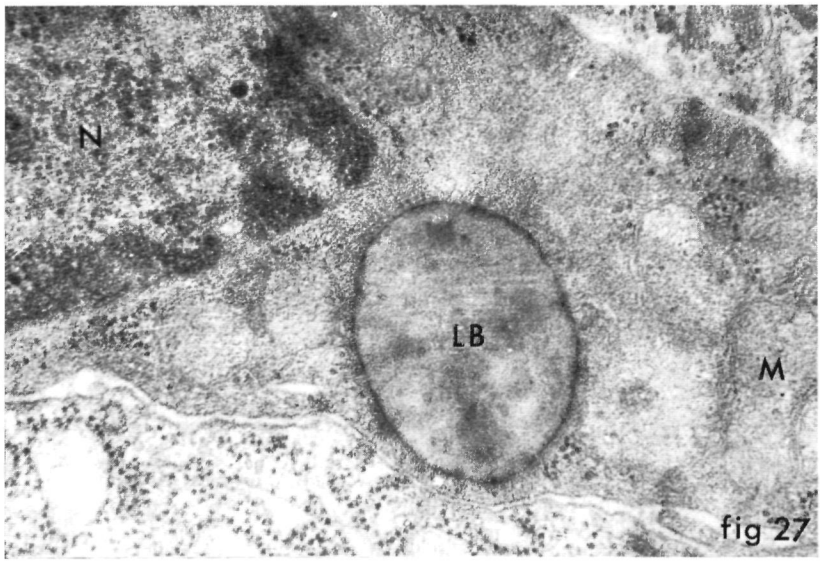


Fig. 27. Lipoid body (LB) in an acinoductular cell. x 32,000.

similarities and are therefore homologous cell types. All three types of cells in the dog pancreas are characterized by a cytoplasm containing very little endoplasmic reticulum, a nucleus with a highly irregular outline, a small Golgi complex and a lipoid body. However, in contrast to the findings of Ekholm et al. (1962) we observed in all three types of cells a rather great number of mitochondria. These findings indicate that these cells most probably have the same function in exocrine pancreatic secretion and a highly active metabolism.

The exocrine pancreas consists of two types of cells, i.e. acinar and ductular cells. The acinar cells have been demonstrated to be quite definitely responsible for synthesis, storage and secretion of the digestive enzymes (Palade et al., 1962). Moreover, there is no evidence showing that these cells are involved in pancreatic fluid and electrolyte secretion.

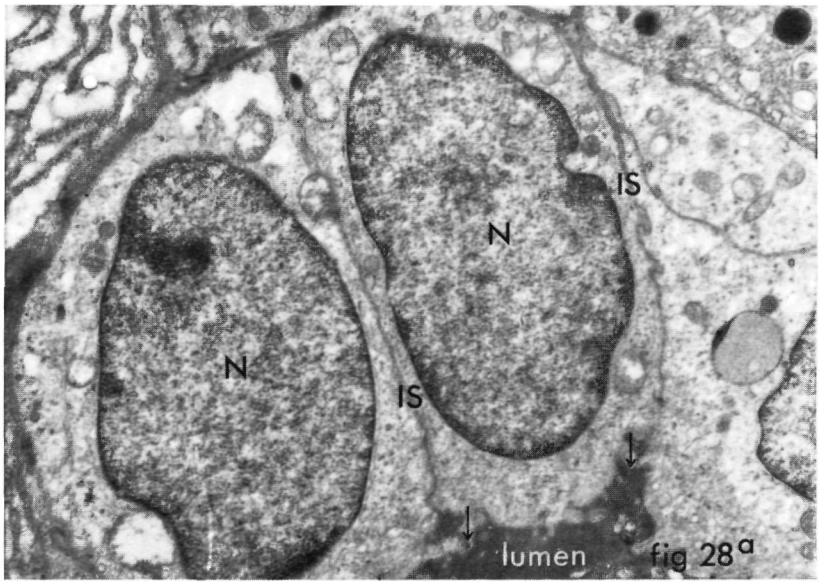
The marked sparseness of the rough endoplasmic reticulum in the ductular cells rules out any large-scale synthesis of exportable enzymes and thus a role in enzyme secretion. An observation favouring a secretory function of the ductular cells is the finding of Becker (1962) that histochemically only the ductular cells possess high carbonic anhydrase activity, which is characteristic for cells involved in fluid secretion (Maren, 1967). These findings strongly suggest that the ductular cells must be responsible for pancreatic fluid and electrolyte secretion. This conclusion is reinforced by our observation that the intercellular spaces of the ductular cells are distended after secretin stimulation, as was also observed in the reabsorbing gall bladder (Kaye et al., 1966). A

careful study of the electron micrographs of the ductular cells of the secretin-stimulated cat pancreas published by Hermodsson (1965) shows marked distention of the intercellular spaces, although the author himself does not mention this point.

The presence of apical blebs, the most characteristic feature of the ductular cells in the pancreas of the rat and cat, has been interpreted as an indication for a secretory activity of these cells (Ekholm, 1962; Hermodsson, 1965). Such apical blebs were never observed in our studies of dog pancreatic tissue. Therefore, it seems unwise to interpret bleb formation as an indication for secretory activity. They may represent fixation artefacts and swelling phenomena due to the use of a non-isotonic fixative solution (Maunsbach, 1966). Characteristic for the dog pancreas is the occurrence of a lipoid body within the ductular cells. The significance of this lipoid body is unknown.

The distention of intercellular spaces, as in the case of the reabsorbing gall bladder, can be interpreted in terms of local osmosis: the membrane of the secretory cells possesses a considerable (Na^+-K^+)-stimulated ATPase activity, which acts as a sodium pump. This pump system secretes Na^+ from the cytoplasm into the intercellular space and so causes locally a high concentration of sodium. In response to the local osmotic gradient thus created, water crosses the membrane and so gives rise to an enlargement of the intercellular space. In this space mixing of water and ions to an isosmotic fluid will take place. The inflow of the water will result in a hydrostatic pressure, which will tend to empty the space content through the narrow opening on the side opposite the tight junctions. As a result of the active secretion of Na^+ across the cell surface into the intercellular spaces, Na^+ will diffuse passively into the cell. Even with an equal distribution of (Na^+-K^+)-stimulated ATPase activity per unit surface a net Na^+ transport into the intercellular spaces would occur, if the cell surface lining these spaces is considerably larger than that between the tight junctions at the luminal side.

The rather surprising aspect of our findings is that the tight junctions closing the distended intercellular spaces are usually found located at the luminal side, while these spaces are open at the basement membrane side. This would indicate a movement of Na^+ and consequently of water away from the ductular lumen, rather than into the lumen. In this connection it should also be noted that the fluid-secreting cells are located downstream from the enzyme-secreting cells, when looking in the general direction of flow towards the intestine. Now the enzymic material secreted in the apex of the acini must have a very high viscosity, because it is a highly concentrated protein solution. Without suitable dilution this viscous secretion could not be expected to flow down the ductules towards the intestine Actually we have noted that secretion obtained during the first hour after mounting the rabbit pancreas,



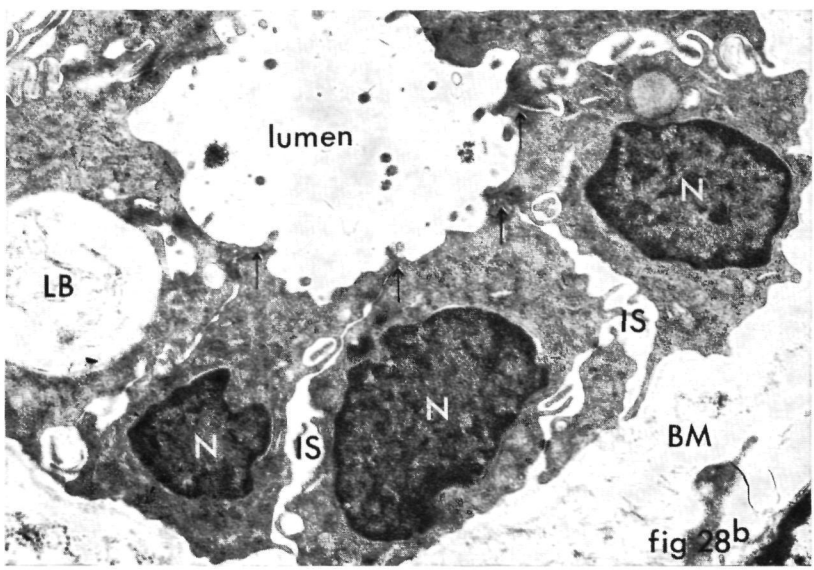


Fig. 28. a: Ductular cells from resting dog pancreas. The intercellular spaces (IS) are very narrow. Arrows indicate the junctional complexes, closing the intercellular spaces at the luminal side. x 15,000. b: Ductular cells from secreting dog pancreas. The intercellular spaces (IS) are now distended, and open at the basement membrane (BM)side. Junctional complexes at the luminal side are again indicated by arrows. x 32,000.

when fluid secretion is only about 40% of that in the third hour and enzyme secretion is double that in the third hour, has a high viscosity. A secretion of water and electrolytes by the ductular cells downstream from the acinar cells would not do much toward diluting the acinar secretion and flushing it down the ductules towards the intestine. More systematic studies about the location of the tight junctions in the ductular cells and the dimensions of the acinus are underway and it is hoped that these results will shed some light on this unresolved aspect of the exocrine secretion of the pancreas.

At any rate, the results reported here indicate that both the ductular and the acinoductular cells are involved in water and electrolyte secretion, while the distended intercellular spaces after secretin stimulation strongly suggest that a local osmosis, as demonstrated in the gall bladder by the physiological work of Diamond (1962) and the electronmicroscopic studies of Kaye et al (1966), must be operating in pancreatic fluid secretion.

SUMMARY

Active sodium transport by means of the (Na^+-K^+)-activated ATPase system appears to play a primary role in the secretion of fluid and electrolytes by organs with a secretory function. The constancy of the sodium concentration in pancreatic juice over widely differing flow rates suggests that a similar mechanism may be the primary and rate-limiting event in exocrine pancreatic secretion. These considerations have led us to a study of the occurrence of the (Na^+-K^+)-activated ATPase system in homogenates of the pancreas and its role in the secretion of both water and electrolytes as well as of enzymes by the pancreas.

The properties of the (Na^+-K^+)-activated ATPase system in dog and rabbit pancreas as well as the properties of the fluid and electrolyte secretory system are investigated. The mechanism involved in the secretion of digestive enzymes by the pancreas is also studied. Finally, the ultrastructural changes of the ductular cells of the pancreas after stimulation by secretin are examined

Chapter I describes our current knowledge of the morphology and physiology of the exocrine pancreas, in as far as relevant to the secretion of fluid and electrolytes and of digestive enzymes.

In Chapter II, our current insight in the mechanism of the cation pump in relation to the (Na^+-K^+)-activated ATPase system is reviewed in detail. Special attention is paid to the role of the pump system in secretory processes.

The methods used throughout our study are described in Chapter III. In addition to determinations of the relevant ions, the assay procedures for Na^+-K^+ ATPase and α-amylase activities are described.

In Chapter IV the enzymatic properties of the (Na^+-K^+)-activated ATPase system of dog pancreas are reported, particularly the activation by sodium and potassium, pH dependence and inhibition by ouabain.

After thus establishing the presence of the enzyme system in dog pancreas, its role in pancreatic fluid and electrolyte secretion in vivo in the dog is studied (Chapter V) Secretin-stimulated fluid secretion in the dog is inhibited by the cardiac glycoside ouabain, with a maximal average flow inhibition of approximately 70% occurring after injection of 45 μg inhibitor/kg body weight directly into the arterial blood supply of the organ. The concentrations of sodium and bicarbonate in the secreted fluid do not change after ouabain administration, while the

potassium and chloride concentrations show small, largely transient increases The molar ratio of Na^+ secreted to ATP hydrolyzed is 1.8, which ratio falls within the range of values (1.8-3 1) previously found for a variety of tissues. While these findings suggest that the (Na^+-K^+)-activated ATPase system plays a part in exocrine pancreatic fluid secretion, the technical limitations of the in vivo approach make in vitro experiments on the isolated organ desirable in order to obtain further evidence. For this purpose the rabbit pancreas, which because of its sheet-like configuration is very suitable for in vitro studies, is used.

First the enzymatic properties of the (Na^+-K^+)-activated ATPase system of rabbit pancreas are studied (Chapter VI). In view of the low relative Na^+-K^+ ATPase activity in rabbit pancreas, the homogenates are pretreated with concentrated urea solution to lower the Mg^{++} ATPase activity and thus raising the relative Na^+-K^+ ATPase activity. The enzyme system is activated by Na^+ and K^+ ions together, while the presence of Mg^{++} is a necessary requirement for activity. The pH optima of the two enzyme activities are also reported. Ouabain does not affect the Mg^{++} ATPase activity, but inhibits the Na^+-K^+ ATPase activity, while at very low concentrations it stimulates the latter enzyme activity. Other cardiac glycosides and erythrophleine also have an inhibitory effect on the (Na^+-K^+)-stimulated ATPase activity. Hexahydroscillaren A, which is an inactive cardiac glycoside, does not inhibit this enzyme activity.

After thus demonstrating the presence of the (Na^+-K^+)-activated ATPase system in rabbit pancreas, its role in fluid and electrolyte secretion by the isolated rabbit pancreas is studied (Chapter VII). Basal pancreatic fluid secretion in vitro is inhibited by various cardiac glycosides and erythrophleine in the same order as the enzyme activity. For ouabain inhibition the pI_{50} is 5.4, the same value as found for the enzyme system. As in the case of the (Na^+-K^+)-activated ATPase system, low ouabain concentrations stimulate flow and hexahydroscillaren A fails to affect fluid secretion by the isolated rabbit pancreas.

The slight flow inhibition by acetazolamide in concentrations fully inhibitory to carbonic anhydrase rules out a primary role of this enzyme in fluid secretion. The strong inhibition of flow by anaerobiosis and Na-azide and the absence of an effect of NaF indicate that fluid secretion is maintained by energy derived from oxidative phosphorylation.

Changes in the Na^+ concentration in the bathing solution lead to proportional changes in Na^+ excretion and almost proportional changes in fluid secretion, which effects are reversible. Ouabain inhibits ^{22}Na and fluid secretion nearly to the same degree, while secretin stimulates both approximately equally. These findings strongly suggest that the sodium pump by means of the (Na^+-K^+)-activated ATPase system, is

the primary and rate-limiting process in pancreatic fluid and electrolyte secretion, and that water follows Na^+ passively and isosmotically.

Chapter VIII deals with pancreatic enzyme secretion in vitro. Ouabain and acetazolamide have no effect on pancreatic enzyme secretion. Anaerobiosis and Na-azide inhibit flow to a larger degree than the secretion of digestive enzymes. NaF does not affect flow, but stimulates enzyme secretion. Incubation of the isolated gland in a low sodium environment has a much smaller inhibitory effect on enzyme secretion than on flow. These results rule out a coupling of enzyme secretion to cation transport. Cyclic AMP and theophylline are potent stimulators of enzyme secretion by the isolated rabbit pancreas. Theophylline also potentiates the stimulatory effect of pancreozymin on enzyme secretion. These results indicate that cyclic AMP is a mediator in pancreatic enzyme secretion.

Electronmicroscopic studies (Chapter IX) reveal that the acinoductular cells and the cells of the intercalary and intralobular ductules exhibit the same ultrastructure. This suggests that they are involved in the same secretory process. After secretin stimulation the intercellular spaces are distended. This finding is compatible with the biochemical evidence of Chapter VII that local osmosis is involved in exocrine pancreatic fluid secretion. This would explain the isotonic character of the secretion of electrolytes and water in the pancreas.

SAMENVATTING

Actief natrium transport blijkt een primaire rol te spelen bij de secretie van water en electrolyten door secreterende organen, waarbij gesteld kan worden dat de natriumpomp identiek is aan het $(Na^+ + K^+)$-geactiveerde ATPase-systeem. De constante natrium concentratie in pancreassap bij sterk uiteenlopende secretiesnelheden doet denken aan een analoog mechanisme bij de secretie van pancreassap. Deze beschouwingen hebben geleid tot een onderzoek naar het voorkomen van het $(Na^+ + K^+)$-geactiveerde ATPase-systeem in pancreas homogenaten en de rol van het enzymsysteem bij de secretie van zowel electrolyten en water als van spijsverteringsenzymen door de pancreas.

Wij beschrijven in dit proefschrift enige onderzoekingen omtrent de eigenschappen van het $(Na^+ + K^+)$-geactiveerde ATPase-systeem in de honde- en konijnepancreas. Tevens hebben wij de eigenschappen van het secretorisch systeem voor water en electrolyten bestudeerd. Ook hebben wij een onderzoek ingesteld naar het mechanisme verantwoordelijk voor de secretie van spijsverteringsenzymen. Tenslotte hebben wij de ultrastructuur van de ductulaire cellen van de pancreas na stimulering door secretine bestudeerd.

Hoofdstuk I geeft onze huidige kennis weer van de morphologie en de physiologie van de exocriene pancreas, voor zover deze betrekking heeft op de secretie van water en electrolyten enerzijds, en de secretie van spijsverteringsenzymen anderzijds.

In Hoofdstuk II wordt het huidige inzicht weergegeven in het mechanisme van de kationenpomp en het verband hiervan met het $(Na^+ + K^+)$-geactiveerde ATPase-systeem. Bovendien is aandacht geschonken aan de functie van het kationenpompsysteem in dierlijke cellen, vooral in verband met secreterende processen.

De methodieken toegepast bij het onderzoek worden beschreven in Hoofdstuk III. Naast enkele chemische bepalingen worden de bepalingsmethoden voor $Na^+ + K^+$ ATPase en α-amylase activiteit behandeld.

De resultaten verkregen uit een onderzoek naar de eigenschappen van het $(Na^+ + K^+)$-geactiveerde ATPase-systeem uit de hondepancreas zijn neergelegd in Hoofdstuk IV, m.n. de activering door natrium en kalium ionen, pH-afhankelijkheid en remming door ouabaine. Na aldus het voorkomen van het enzymsysteem in de hondepancreas te hebben aangetoond, is een onderzoek ingesteld naar zijn rol bij de secretie van water en electrolyten door de pancreas in vivo (Hoofd-

stuk V). De door secretine gestimuleerde secretie van pancreassap wordt geremd door ouabaine, dat is ingespoten in de arteriele bloedvoorziening van het orgaan. Na toediening van 45 µg ouabaine/kg lichaamsgewicht wordt een maximale secretieremming gezien van 70%. De natrium en bicarbonaat concentraties in het secreet blijven onveranderd, terwijl de kalium en chloride concentraties tijdelijk toenemen. De molaire verhouding van gesecreteerde Na^+ tot gehydrolyseerde ATP bedraagt 1.8. Deze verhouding valt binnen het bereik van de waarden, die eerder gepubliceerd zijn voor diverse andere weefsels (1.8-3 1). Hoewel deze resultaten pleiten voor een rol van het ($Na^+ + K^+$)-geactiveerde ATPase-systeem bij de secretie van pancreassap, zijn in vitro experimenten met het geisoleerde orgaan wenselijk, vanwege de beperkingen van het onderzoek in vivo. Vanwege zijn geringe dikte is de konijnepancreas zeer geschikt voor dit doel

In Hoofdstuk VI worden de resultaten van het onderzoek naar de eigenschappen van het ($Na^+ + K^+$)-geactiveerde ATPase-systeem uit de konijnepancreas beschreven. Aangezien de ($Na^+ + K^+$)-gestimuleerde ATPase activiteit laag is worden de pancreas homogenaten voorbehandeld met een geconcentreerde ureumoplossing. Hierdoor neemt de Mg^{-+} ATPase activiteit sterk af, en de ($Na^+ + K^+$)-gestimuleerde ATPase activiteit sterk toe. Het enzymsysteem wordt door natrium en kalium ionen tezamen geactiveerd, terwijl Mg^{++} ionen noodzakelijk zijn voor de activiteit. Daarnaast hebben wij ook de pH-optima van beide ATPasen bepaald. Ouabaine heeft geen effect op het Mg^{++} ATPase, doch blijkt de ($Na^+ + K^+$)-gestimuleerde ATPase activiteit te remmen, terwijl lage concentraties van het hartglycoside een stimulerend effect hebben op laatstgenoemde activiteit Andere hartglycosiden en erythrophleine blijken ook remmend te werken op de enzymactiviteit. Hexahydroscillareen A, een onwerkzaam hartglycoside, remt de enzymactiviteit niet.

Hoofdstuk VII betreft een onderzoek naar de rol van het ($Na^+ + K^+$)-geactiveerde ATPase-systeem bij de secretie van water en electrolyten door de geisoleerde konijnepancreas. De basale secretie in vitro wordt geremd door verscheidene hartglycosiden en erythrophleine. Voor de remming van de secretie door ouabaine wordt een pI_{50} van 5.4 gevonden, gelijk aan die voor het enzymsysteem. Lage concentraties ouabaine daarentegen stimuleren de secretie van pancreassap in vitro Hexahydroscillareen A heeft, evenals op de enzymactiviteit, geen effect op de vloeistofsecretie.

De relatief kleine remming van de vloeistofsecretie door acetazolamide in concentraties die het koolzuur anhydrase volledig remmen, sluit een primaire rol van dit enzym in vloeistofsecretie uit. De sterke remming van de vloeistofsecretie door anaerobe condities en Na-azide en de afwezigheid van een effect van NaF, wijzen erop dat de vloeistofsecretie onderhouden wordt door energie verkregen uit de oxydatieve

phosphorylering. Wanneer de geïsoleerde konijnepancreas geïncubeerd wordt in een natrium-arm medium heeft dit tot gevolg dat de Na^+ secretie evenredig en de vloeistofsecretie bijna evenredig dalen Toevoeging van het ontbrekend ion aan de incubatievloeistof geeft een volledig herstel van de vloeistofsecretie. Ouabaïne blijkt voorts de ^{22}Na- en vloeistofsecretie in vrijwel gelijke mate te remmen, terwijl secretine beide secretorische processen vrijwel gelijkelijk stimuleert. Deze resultaten rechtvaardigen de conclusie, dat ook bij de secretie van pancreassap een primaire rol toekomt aan de secretie van natrium door middel van een ($Na^+ + K^+$)-geactiveerde ATPase kationenpomp en dat water passief en isosmotisch het Na^+ volgt.

In Hoofdstuk VIII zijn de resultaten verkregen uit een onderzoek naar het mechanisme van enzymsecretie in vitro neergelegd. Ouabaïne en acetazolamide blijken geen effect te hebben op de enzymsecretie. Onder anaerobe condities wordt de vloeistofsecretie in veel sterkere mate geremd dan de enzymsecretie. Dit blijkt ook het geval te zijn na toevoegen van Na-azide aan het incubatiemedium. NaF echter beïnvloedt de vloeistofsecretie niet, maar blijkt de enzymsecretie te stimuleren Bij incubatie van de geïsoleerde konijnepancreas in een natrium-arm medium wordt de enzymsecretie veel minder geremd dan de vloeistofsecretie. Deze resultaten rechtvaardigen de conclusie dat de secretie van spijsverteringsenzymen door de celmembraan niet gekoppeld is aan het transport van electrolyten en vloeistof Cyclisch AMP en theophylline blijken de enzymsecretie te stimuleren. Bovendien potentieert theophylline het stimulerend effect van pancreozymine op de enzymsecretie Deze resultaten doen vermoeden dat het cyclisch AMP een rol speelt bij de secretie van spijsverteringsenzymen.

Een electronenmicroscopisch onderzoek (Hoofdstuk IX) toont aan dat de acinoductulaire cellen en de cellen van de intercalaire en intralobulaire ductuli een sterk overeenkomende structuur hebben. Deze structuur maakt het waarschijnlijk dat deze cellen betrokken zijn bij dezelfde secretorische functie. Na stimulering door secretine blijken de intercellulaire ruimten tussen de epitheelcellen verwijd te zijn, hetgeen erop wijst dat locale osmose, zoals o.a. in de galblaas is gevonden, een rol speelt in de exocriene secretie van de pancreas. Daarmee zou het isotone karakter van de secretie van electrolyten en water verklaard zijn.

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STELLINGEN

I

Bij een verder onderzoek naar het mechanisme van de secretie van spijsverteringsenzymen door de pancreas verdient het aanbeveling aandacht te schenken aan de mogelijke rol van het phospholipase A

II

Het is niet verantwoord dat McConnell et al. suggereren dat hun preparaten intacte staafzakjes (photoreceptor discs) zouden bevatten. Hun experimenten hebben danook eerder betrekking op gefragmenteerde fotoreceptor membranen dan op fotoreceptor fragmenten

D G McConnell, C N Rafferty en R A Dilley, J Biol Chem, *243* 5820, 1968

III

De door Konishi en medewerkers beschreven verandering van de negatieve cochleaire potentiaal na perfusie van de perilymfatische ruimte met Ringer oplossing, is zeer waarschijnlijk het directe gevolg van de opgeloste zuurstof in de perfusievloeistof en niet van een drukverhoging.

T Konishi, E Kelsey en G T Singleton, Acta Otolaryng, *64* 107, 1968

IV

De sequentie-analyse van peptiden zal in de nabije toekomst aanzienlijk vereenvoudigd kunnen worden door toepassing van massaspectrometrie.

J P Kamerling W Heerma en J F G Vliegenthart, Organic Mass Spectrometry *1* 351, 1968

V

De noodzaak van samenwerking tussen artsen (huisartsen en specialisten) en het maatschappelijk werk wordt algemeen erkend In de praktijk wordt deze samenwerking echter aanzienlijk bemoeilijkt door het verschil in werktempo, divergente verwachtingen omtrent resultaten en een wederzijdse gebrek aan waardering.

VI

Indien de stijging van de alkalische phosphatase activiteit van het serum bij patienten met longinfarcering een gevolg zou zijn van fibroblasten woekering, dan zou men dit verschijnsel eveneens moeten zien optreden bij andere longprocessen, die gepaard gaan met weefselverval en organisatie Het verschijnsel als zodanig mag danook niet als specifiek voor longinfarcering beschouwd worden.

J H Dijkman en P W C Kloppenborg, Acta Med. Scand., *180* 273, 1966

VII

Pancreozymine is in staat zowel de secretie als de *de novo* synthese van spijsverteringsenzymen te stimuleren.

P D Webster en P T Malcolm Am J Physiol., *211* 157, 1966
S S Rothmann en H Wells, Am J Physiol., *213* 215, 1968

VIII

Interferon geproduceerd door cellen van een bepaald diersoort vertoont een specifieke beschermende activiteit. Deze soortspecificiteit blijkt echter niet altijd absoluut te zijn.

J Desmyter, W E Rawls en J L Melnicke, Proc Nat Acad Sci., *59* 69, 1968

IX

Studiebegeleiding voor eerstejaars medische en tandheelkundige studenten is een bittere noodzaak en dient bij voorkeur op min of meer schoolse wijze te geschieden.

X

Binnen het kader van vernieuwingsdrang en progressiviteit, markante karakteristieken van deze tijd, dient zeker de gedachte om alle carnavalsfestiviteiten te verplaatsen naar een warmer jaargetijde, overwogen te worden.